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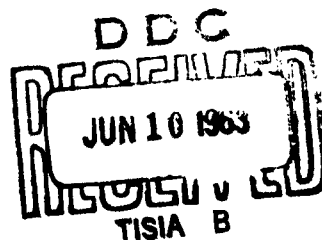
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# COMMISSION ON EPIDEMIOLOGICAL SURVEY

ARMED FORCES  
EPIDEMIOLOGICAL BOARD



ANNUAL REPORT - 1962

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**COMMISSION ON EPIDEMIOLOGICAL SURVEY**

**Armed Forces Epidemiological Board**

**ANNUAL REPORT**

**1962**

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COMMISSION ON EPIDEMIOLOGICAL SURVEY

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Theodore E. Woodward, M.D., Director

Leighton E. Cluff, M.D., Associate Member

Dan Crozier, M.D., Associate Member

Fred R. McCrumb, M.D. Associate Member

### THE DIRECTOR'S REPORT

One meeting of the entire Commission was held on September 6 and 7, 1962, and several ad hoc meetings for discussion of specific problems were held during 1962-63. On the occasion of the Commission meeting, the discussion focused on the problem of plague. In addition to the members of the U. S. Army Medical Unit and the U. S. Army Biological Laboratories at Fort Detrick, Dr. Marcel Baltazard, Director of the Pasteur Institute, Teheran, Dr. K. F. Meyer, Director Emeritus of the George William Hooper Foundation and Dr. R. Pollitzer entered into the pertinent discussions. The details of the respective reports are given in the appended annual report.

Dr. William Lawton discussed plague antigens, stressing that V and W antigens can be separated, that the V fraction rather than the W antigen is protective for mice. Studies revealed that antitoxin does not protect against death and that an endotoxin-like effect may be associated with immunity although it is not known if endotoxin alone will protect.

Major John D. Marshall reported on plague vaccine trials in mice, indicating varying activities with the numerous vaccines used. Immunized animals were observed to die late, up to 21 days, when plague lesions were noted. No specific conclusions regarding the data were drawn.

Mr. Werner Janssen described the phagocytic mechanisms involved in plague infections. Plague bacilli are phagocytized in the liver and spleen and both sensitive and resistant strains are taken up in fixed phagocytic cells. Pasteurella pestis has been observed to form spheroplasts inside cells. Polymorphonuclear leukocytes phagocytize plague bacilli; bacteria released from cells by electrical disruption were found to be viable.

Dr. Raymond Randall and his group working at Forest Glen have detected the presence of antibody by micromethods, using a modified HI tannic acid test sensitizing red cells with Fraction I. A system using Bentonite particles is under study. The test may have application for field studies.

Dr. K. F. Meyer presented the results of systematic observations of immunization procedures against plague carried out at the Hooper Foundation over several years. Very few side effects result from immunization when less than 3 billion plague bacilli are administered. The interscapular site is regarded as an ideal area. Dr. Meyer stressed the need for booster immunization after an initial basic series. Adjuvants may be useful which might permit the incorporation of smaller amounts of antigen.

Dr. Meyer recounted the vivid, historical features of plague in his inimitable manner by describing the pertinent epidemiologic, pathologic and clinical manifestations of this age-old disease. He spoke of the doughy tissue edema surrounding the bubo and urged that lymph be taken

from this area for early diagnostic study. He was unable to state the number of bacilli required to produce a bubo or the number of plague bacilli which are regurgitated by fleas. Doctor Meyer stressed the need for information regarding the mechanism of death in plague. One is unable to draw conclusions from animal data as to whether the soluble murine toxin, which is high in protein, or the lipoid toxin is responsible for death. The mechanisms responsible for secondary shock which characterize disease in animals are unknown. Cases of pneumonic plague in California were observed to have "bubonic tonsillar plague."

Dr. Marcel Baltazard described his studies of the ecology of plague which have been conducted in Iran and India over many years. A report pertinent to Doctor Baltazard's discussion is reprinted in this annual report. His field studies of plague infection in domestic rodents, commensal rodents and wild rodents lead him to believe that the true cycle of plague is latent, or quiet, such as that which is observed in resistant animals. He compares it to brucellosis which is typified by no interhuman transmission. Plague is regarded as a disease of wild rodents and their fleas occurring in nature like typhus fever and recurring at times of stress or war. Although global plague is now quiescent he regards it as the disease of the future. Doctor Baltazard described his field studies of the spread of plague in the common gerbil, Meriones, and his concepts concerning "pockets" of plague in nature. These are described in the reprint.

Major William Sawyer described studies of the influence of the duration of the airborne state upon the infectivity of aerosolized Pasteurella tularensis for Macaca mulatta and for man. The results indicate that airborne organisms lose infectivity more rapidly than they do the ability to multiply on appropriate culture media. Such dissociation of infectivity from viability was apparent when the organisms had been airborne 120 or more minutes.

Colonel Dan Crozier related plans for an intensive effort by Medical Unit personnel aimed at defining dose response parameters and the pathophysiology of illness induced by staphylococcal enterotoxin.

The typhoid studies of Doctors Sheldon E. Greisman, Richard B. Hornick, and Frank A. Carozza, Jr. suggest that the endotoxin of Salmonella typhosa is responsible for the clinical manifestations of typhoid fever. Pertinent physiologic and immunologic investigations suggest that a humoral factor may alter the reaction of human subjects to endotoxin. This is based on recent passive transfer studies. The conventional and purified typhoid vaccines have not shown significant protection in volunteers who have been previously immunized and challenged with large doses of S. typhosa (Quarles strain). These vaccine trials are continuing.

Theodore E. Woodward, M.D.  
Director

April 15, 1963

## EXECUTIVE SESSION

### COMMISSION ON EPIDEMIOLOGICAL SURVEY

WALTER REED ARMY INSTITUTE OF RESEARCH

1330 hours, September 7, 1962

The Chairman made several brief comments and announcements to open the Executive Session. Appreciation was expressed to Colonel Dan Crozier for his excellent direction of the Medical Unit, in developing a commendable scientific program and for his promotion of an excellent esprit de corps among the various scientific groups. During the summer Colonel Crozier and the Chairman met with Drs. Smadel and Shope at Fort Detrick for the purpose of discussing the in-service program of the Medical Unit. This was a very productive session, which led to interesting discussions and pertinent comments pertaining to the general scientific program. Such small Ad Hoc group meetings are useful and it is contemplated that more will be held throughout the year in order to take advantage of the excellent advice by various Commission members. During the coming year, several small meetings to be organized by Colonel Crozier will be held; the Commission will conduct one large meeting preferably as soon after Labor Day as possible.

The Annual Report of the Commission has been distributed to members and various interested persons. An article has been prepared by Colonel Crozier and the Chairman as a Resume of Activities of the Commission. This will appear in "Military Medicine."

The paper entitled "Live and Killed Tularemia Vaccines: Evaluation in Animals and Man," by H. T. Eigelsbach, W. D. Tigertt, S. Saslaw, and F. R. McCrumb, Jr., presented at the 1962 Army Science Conference was cited for a special award. The authors received a commendation and a monetary award.

Doctor Marcel Baltazard, Director of the Pasteur Institute, Teheran, attended the current meeting of the Commission. His contributions to our knowledge of plague are well known and his comments stimulated all present. Dr. Baltazard's trip to the United States and return to Iran was supported by Commission funds; his visits in the United States to Baltimore, California, Seattle, and Hamilton were supported by the Institute for Allergy and Infectious Diseases of The National Institutes of Health. Dr. Baltazard is an outstanding emissary of France and Iran.

The Chairman mentioned that Colonel Gray and Lt Colonel Beisel of the Medical Unit have initiated metabolic and nutritional studies on various infectious diseases; these will be coordinated in part with the typhoid investigations in Baltimore.

Additional membership in the Commission was discussed. After some comment it was concluded that the Commission would not be enlarged.

Colonel Crozier discussed fiscal and scientific problems related to the Medical Unit's program.

1. The program for 1962 provides the sum of \$2,195,000.00 followed by steady yearly increases until 1967. These funds are for in-house and extramural activities.

2. Studies on staphylococcal enterotoxin are well underway. It is hoped that within 12 to 18 months a suitable assay system will have been developed, and the effects of the enterotoxin will have been defined. This request came to the Commission from a DOD level. Certain specific protocols must be approved by DOD and will be submitted in general and specific terms. Dr. Smadel suggested that requests be submitted for initial approval and, at appropriate times supplemental approval be obtained for specific projects.

3. Colonel Crozier announced that a plague program will be initiated in the Forest Glen Laboratory as soon as necessary renovations are completed. Dr. Smadel suggested that the local health officer be informed of the plans to engage in plague research, reminding him that this will represent reactivation of an old approval.

The Chairman announced that after detailed discussions between Dr. Dingle and various Commission members it was decided that he should not assume responsibility for full-time direction of the Commission. For reasons of his health and responsibilities in Cleveland, it seemed advisable that he not undertake new activities. During the May 1962 meeting of the Armed Forces Epidemiological Board, Drs. Shope, MacLeod, Dingle, and Woodward, and Colonel Crozier discussed the problem of civilian leadership. It was suggested that Dr. Fred R. McCrumb, Jr. devote much of his time to current affairs of the Commission and serve as scientific advisor of the Medical Unit. University of Maryland authorities have expressed interest in this proposal, but stress that laboratory and hospital bed facilities in Baltimore are now totally inadequate for conduct of the present work supported by the Commission. The University of Maryland has submitted a letter of intent to Brig General Robert E. Blount, Commanding General, Medical Research and Development Command, U. S. Army, which indicates the interest in having Dr. McCrumb serve in this senior advisory capacity to the Commission. At the same time a preliminary request was made for funds sufficient to permit the building of adequate laboratory and bed facilities to insure continuation and expansion of the studies concerned directly with the Commission and the Medical Unit's mission. On completion of appropriate preliminary discussions the Commission will be informed of the precise requests of the Maryland Group. At this time the Commission members will be urged to comment.

The meeting was adjourned at 1600 hours.

## REVIEW OF PLAGUE

### EPIDEMIOLOGY OF PLAGUE

Marcel Baltazard, M.D.\*

(Reprinted from WHO Chronicle 14(11):419-426, 1960.)

The first modern epidemiological studies of natural plague suggested that the disease was due solely to highly susceptible rodent species, because of the spectacular epizootics among these species. The rodents concerned were either domestic ones, headed by the rat, the main cause of human plague, or wild or field species, such as the gerbils in South Africa and the Sciuridae in the Asian or eastern European foci (tarabagans, susliks) and in the Americas (ground squirrels, prairie dogs). It is true that, in these rodent populations, plague always had a tendency to die out because of the very violence of the epizootics. That this disappearance was complete was suggested by the negative results obtained in all investigations of these populations during the inter-epizootic periods. However, the continual recrudescence of the disease at the same places in wild foci, and its never-ending persistence in certain rural or urban foci, where the rat was thought to be the only rodent involved, seemed proof of its perpetuation of these highly susceptible species.

#### A NEW THEORY

In 1952, the Institut Pasteur de l'Iran, on the basis of its researches in Kurdistan, introduced the new idea that highly resistant species play a part in plague epidemiology. The research workers of the Institute enunciated the following basic principle. Any species exterminated by a disease cannot be the reservoir of this disease, or, in other words, the true reservoir of a disease must be sought not among the most susceptible species but among those whose natural resistance shows them to be the best adapted to the disease. This principle, which may appear self-evident, was in fact directly opposed to accepted ideas, particularly in the case of plague, where resistance to infection had always been considered as a factor limiting its spread.

According to the new theory, the true reservoirs of infection were the highly resistant rodent species and the susceptible species were only temporary victims. Once these susceptible species, and in particular the rat, were regarded as unable to perpetuate the disease, the whole problem of plague epidemiology had to be reconsidered, especially in the case of the so-called "pure murine" foci (India, Java, Madagascar, Kenya, etc.).

#### INVESTIGATIONS

The new concept found a very wide audience. In Kenya, R. B. Heisch was the first to prove that in one of the most reliably established "pure murine" foci anywhere in the world, Rattus rattus played no part in

\* Director, Institut Pasteur de l'Iran.

maintaining the disease and autonomous wild rodent plague existed, based on highly resistant species. In the USA, investigations in the inveterate western foci were directed, at the instance of K. F. Meyer, towards species resistant to the infection and rapidly revealed the primary role played by some of them (Microtinae, Cricetinae). In the USSR, despite the firmly held belief that marmots and Citellus (susliks) played a predominant role, a number of workers turned their attention to the resistant species. Gerbillinae, including several species of Meriones and the gerbil Rhonbomys opimus, Cricetinae (Cricetulus), Microtinae (Alticola and Microtus) and even Lagomorpha (Ochotona) were then incriminated. These species, which for the most part had already been recognized by Soviet workers as being subject to infection and whose resistance was known or had recently been demonstrated, are now considered to play the principal role in most areas of permanent infection.

Finally, WHO gave the Institut Pasteur de l'Iran an opportunity to extend its investigations by entrusting it with a series of epidemiological surveys requested by the Governments of India, Java, Iraq, Syria and Turkey. These surveys took place from 1954 to 1957, in collaboration with national teams, the Institute meanwhile continuing its work in Iranian Kurdistan. This group of investigations, in conjunction with those commenced in Morocco in 1941 by the Institut Pasteur du Maroc, under the leadership of Georges Blanc, covered practically all aspects of plague throughout the world and made it possible to solve a number of controversial questions relating to the epidemiology of the disease.

#### PERMANENT FOCI

The Institut Pasteur de l'Iran had originally announced that the Kurdistan focus was "a focus of pure Meriones plague." Classifications by taxonomists specializing in the Meriones genus indicated the presence of three species only, which were shown experimentally to be highly resistant to plague. The workers of the Institute were thus led to carry to its extreme limit their theory of the predominating part played by resistance to the disease, by concluding that "plague can thus maintain itself only in highly resistant rodents."

Further researches in Kurdistan were to show definitely that serious confusion existed in the classification of the genus Meriones, which had already been repeatedly modified by the experts. The Institute undertook the study and reclassification of the Meriones of Kurdistan, with help from biologists, cytologists and taxonomists. This reclassification revealed profound differences between the species of this genus, superficially so homogeneous. One of these differences was concerned precisely with susceptibility to plague. Four (and not three) species existed in Kurdistan, and were found throughout the area investigated, which had been extended in the meantime to Iraq, Syria and Turkey. Only two of these species, M. persicus and M. libycus, possessed the resistance regarded as the sine qua non for the continued persistence of the disease; the two other species defined in the course of reclassification, M. vinogradovi and M. tristrami, were on the contrary highly susceptible to plague. In each of the permanent microfoci

studied in Kurdistan, there were at least one of the resistant species and at least one of the susceptible species, closely mingling in their habitat. Thus, while other workers were recognizing the basic role played by resistant species in plague among susceptible species in inveterate foci, the Institut Pasteur de l'Iran came to the conclusion that the presence of susceptible species able to start epizootic infection afresh among resistant species was necessary to maintain such foci. The main cause of the perennial nature of plague in inveterate foci could thus be defined as the presence of a resistant-rodents-susceptible-rodents complex.

However, research in temporary foci was to show that this was not sufficient cause in itself. The mere presence of resistant species could bring about only temporary persistence. For perennial plague to occur, it was necessary to have a dense population of very highly resistant rodents capable of surviving the most violent epizootic plague in large numbers - i.e., sedentary rodents, able to maintain the infection in fleas, in deep, permanent burrows with a favourable microclimate.

#### TEMPORARY FOCI

Encountered all over the world and throughout history, these foci multiplied at the time of the "modern pandemic." They were characterized by a more or less prolonged period of persistence following the introduction of plague. This persistence, although suggesting that the disease was permanently implanted, was nevertheless followed by its complete disappearance. Responsibility for this temporary persistence had been attributed in most of these foci to commensal rats, and its relatively long duration, as opposed to the usual brief duration of the historical onslaughts of the disease, had been regarded as due to not very clearly specified climatic or local factors.

The survey carried out by the Institut Pasteur de l'Iran in Mesopotamia showed that, despite a history marked by frequent reappearances of the infection, the focus there was of the temporary type. Similarly, surveys in India and Java proved that the foci there, despite the uninterrupted persistence of plague for half a century, were also only temporary ones from which the disease would no doubt disappear in the very near future.

In all these foci, classified as "pure murine" foci, research showed that the domestic rat played no part at all in the persistence of plague in rural areas and proved that this persistence was brought about by certain species of field rodents. However, these species (Tatera indica in India and Mesopotamia, Rattus exulans in Java) had not the resistance to plague or the density and sedentary nature required to create permanent foci of infection. The epidemiological maps showed, and this was confirmed by the investigations, that plague was continually on the move in rural areas. It rarely persisted for more than a year at any one place, and continued for several years only in exceptional cases. This temporary persistence existed only in areas where resistant species predominated. In Java, for example, infection persisted only in regions where R. exulans was predominant; when plague broke out on the plains in rice-growing areas where only highly susceptible species were to be found, it rapidly subsided.

Phenomena of the same nature (intervention of a resistant species) would seem to explain the persistence of plague in certain ports, as opposed to its usual brief duration in inland towns. The presence in these ports, in addition to R. rattus, of the species R. norvegicus, which is considered to be naturally resistant to the infection and is not found in inland towns, might play a part in this persistence. Finally, the data on persistence in ports had, for the most part, been falsified by the frequent reintroduction of infection, particularly by shipping, during the period when they were established.

#### SPREAD OF INFECTION

The traditional theory according to which the rat alone was responsible for the genesis of human plague offered no satisfactory explanation of the way in which the infection advanced from village to village in India and Java<sup>1</sup>, and, in general, everywhere where plague had appeared and had spread in rural areas. Indeed, ever since the beginning of the century, investigations had shown the extraordinary sedentary nature of R. rattus whose area of movement was restricted and never extended from village to village. The "passive transport" of rats, which was known to be responsible for the spread of plague along railways or navigable rivers, was suggested as an explanation. However, systematic examination of peasants' carts and even of baskets carried from place to place soon showed that such "transport" did not occur in rural areas. There remained the somewhat fanciful suggestion that infected rat fleas were carried from village to village in clothing or objects and produce. Investigations in India and Java showed, however, that this occurred only rarely.

It was the discovery of field rodent infection in these foci that eventually explained the slow, regular advance of rural infection. Research showed that plague moved forward step by step, borne by field rodents, from field to field, from burrow to burrow, in thin epizootic trails winding across the countryside, infecting village rats in passing, and thus setting off murine epizootics which led in turn to human infection. The sporadic nature of infection in villages, i.e., the fact that in the midst of infected territory many villages remained free from the disease, was due to the capricious nature of these epizootic trails and not, as had been suggested, the chance small-scale "transport" of infected fleas by man.

#### THE SEASONAL NATURE OF PLAGUE

Another point which remained obscure in the epidemiology of the disease was the very curious phenomenon of "plague seasons": in India, the incidence of plague drops greatly every year at the beginning of the hot season and rises again only after the end of the monsoon. Long and difficult experimental work carried out by leading experts under varying climatic conditions had shown that heat, particularly when combined with drought, influences the multiplication of fleas, their infection by plague and their vector potential. This explanation, however, did not fit in with certain facts: in certain countries, as for example Java, under climatic conditions practically identical with those obtaining during the monsoon season in India, plague was continuously present; in India itself, moreover, although plague declined considerably in the off-season, it nevertheless continued to exist both in urban and in rural areas.

The investigations of the Institut Pasteur de l'Iran showed, in fact, that in the rural areas it was only the spread of plague and not the disease itself which came to a standstill at the end of each spring. In the north of India, practically no more villages became infected later than the beginning of June. Village plague being usually of brief duration, infection had by then died out in the villages infected at the beginning of the season, and there was accordingly a rapid decrease in the number of plague-stricken villages. Observation showed, however, that in these villages the infection followed a "normal" course despite the heat and drought. The number of rats and fleas, and the incidence and duration of murine and human plague were apparently the same there as at the height of the season.

Research indicated that this seasonal phenomenon depended primarily on the field rodent factor: from May onwards all species of rodents in the fields commenced aestivation, closing themselves in their burrows and living on stored food reserves. Thus the epizootic ceased to advance in the fields and, at the same time contamination of village rats came to an end. When the field rodents again became active, that is in mid-October when the monsoon floods had dried up, the epizootic revived in the fields and murine, followed by human, plague soon reappeared in the villages.

It is possible that if a study were to be made of the movements of rodents in towns during the hot season, it might reveal something of the same nature which would explain the seasonal cessation of the epizootic in urban areas.

#### INTERSEASONAL PERSISTENCE (CARRY-OVER)

This seasonal phenomenon raised another question: where and how did the infection persist during the "off-season" period which lasts for nearly five months? As regards urban plague, the question seemed to have been solved: at the beginning of the century the Plague Research Commission which worked in Bombay for a whole year (October 1905-September 1906) had shown that, although rat plague became rare during the off-season, it nevertheless did not cease to exist; studies by Indian research workers in the large ports subsequently confirmed this. In the rural areas, the degree of persistence of plague (human cases, murine mortality) in the few villages where the disease appeared at the end of the season and was able to maintain itself throughout the off-season was considered sufficient to ensure the carry-over of plague from one season to the next.

The investigations of the Institut Pasteur de l'Iran showed that in the rural areas, even at the height of the infection, this carry-over phenomenon was too rare and scattered to explain the large number of points where plague revived at the beginning of the following season. Moreover, an epidemiological chart, covering four so-called "endemic" districts over the previous thirty years, indicated large areas each year where no village showed the carry-over phenomenon; nevertheless, plague reappeared in these areas at the beginning of the next season. For the most part, it reappeared not in the villages which were the last to be infected at the end of the previous season, but in villages nearby or even a few kilometres

away, in areas which had remained free from infection during the preceding season. Finally, investigations in several villages revealed a high murine mortality rate, which confirmed the extreme rapidity with which the infection normally disappears in the case of the rat.

These investigations also showed the part played by field rodents. The gerbil, Tatera indica, which was the most common of these rodents in the plague area, resisted infection in sufficient numbers in the territory attacked by the epizootic to maintain flea infection during the hot season in its deep, closed burrows, where it was sheltered from unfavourable climatic conditions. Study of the regions flooded each year by the monsoon confirmed the role of this rodent: at the beginning of each season, plague first reappeared in these regions in villages situated on higher land which could not be flooded and where the gerbil burrows had escaped destruction.

Giving this explanation of the persistence of rural plague in which the domestic rat played no part, the research workers of the Institut Pasteur de l'Iran suggested that, in urban areas, the rat might well be able to find conditions lacking in the villages, in respect of numbers and distribution, breeding places protected against climatic conditions (cellars and drains), and the presence of populations or individuals resistant to the infection.

#### EPIDEMIC DEVELOPMENT OF PLAGUE

Immediately after Simond's discovery of natural plague in wild rodents, the work of the Plague Research Commission in India and of the Dutch in Java, confirmed by numerous investigators all over the world, firmly established the theory that the rat alone was involved in the genesis of human plague. Every case of human bubo-septicaemic plague was thus due to the bite of fleas from rats which had died of the disease. However, this theory did not explain everything. Although it was indisputably true for modern plague, in the form then prevalent in India or Java, for example, the fact remained that in those countries the infection was endemic rather than epidemic in nature, and spread slowly unlike the sudden, explosive outbreaks of the past. On the discovery, somewhat later, of the first foci of wild rodent plague where rats were not involved, investigations showed that human plague was rare or exceptional in these foci. It thus seemed certain that bubo-septicaemic plague epidemics did not occur in the absence of the rat, the only rodent able to introduce Xenopsylla cheopis, which experimental research has shown to be the best vector of the disease, into the human habitat.

The studies of the Institut Pasteur du Maroc in 1941, in a focus where human plague was present in its "historical" explosive form, were to reveal the role played by human ectoparasites in the development of epidemics. Nevertheless, the presence of the rat in this Moroccan focus, the simultaneous existence of a violent murine epizootic, and the presence in houses, in the very clothing of persons who had died from plague, of infected X. cheopis led most epidemiologists to dispute the findings of the Moroccan workers.

The first publications of the Institut Pasteur de l'Iran raised anew the question of interhuman transmission in regard to the Kurdistan plague focus, a wild rodent focus without rats where there had nevertheless been epidemics of bubo-septicaemic plague.

Further research by this Institute was to provide a solution for the problem. It confirmed the extreme rarity, if not absence, of human ectoparasites in rural areas in India as in Java. At the same time, however, it showed that plague was not - and in fact never had been - of an epidemic nature in the particular environment concerned. The history of the human infection, which was studied in a large number of villages, showed that plague caused only a series of spaced, sporadic cases there, and that these were few in number at any one time. The epidemic aspect of plague in India and Java was only a statistical one, the systematic addition of cases by canton, district, province, etc., giving enormous figures in periods when the disease was distributed over a wide area.

On the other hand, investigations in the rural foci of Syria, Turkey and Iraq, where human bubo-septicaemic plague had on many occasions taken on the same "historical" epidemic aspect as in Iranian Kurdistan, revealed the same complete absence of the rat and the same high density of human ectoparasites. Proof of interhuman transmission in these foci was obtained in Iran where, during a village epidemic, the plague bacillus was isolated from specimens of Pulex irritans captured in the houses of persons who had died from bubonic plague ten days beforehand.

The controversy regarding the way in which plague becomes epidemic was thus complicated by the application of the word "epidemic" to two different phenomena. The creation of the word "anademic" was suggested to designate the addition of sporadic cases as opposed to the multiplication of cases by the process of interhuman transmission leading to an epidemic.

#### THE DECLINE OF PLAGUE

The decline of plague throughout the world over the past 15 years has been large scale and rapid, though this in no way means that plague can be written off. It marks, however, the close of a unique period in the history of plague - the period of the "modern pandemic" in which the invention of the steamship enabled the disease to spread throughout the world. Today's situation reflects the success of a gigantic attempt - the first that could really be called "international" - to make good one of the most tragic consequences of human "progress."

From the beginning of the century, the successive sanitary conventions imposed increasingly heavy obligations on the ever growing number of countries bound by them. Measures involving enormous expenditure of effort and money were continued for years with a tenacity all the more meritorious in that the results were always disappointing; these measures included the search for and systematic examination of rats in ports and on vessels, the destruction of refuse, the separation of drains in ports, attempts to prevent the embarkation and disembarkation of rats and, in particular, the employment of rat poison and the periodic fumigation of ships.

It was the application of "rat-proofing" that finally led to the gradual elimination of the rat from shipping. The value of this method, conceived and applied in the USA as early as 1907 was only officially recognized in 1926, when the Paris Conference introduced deratting exemption for rat-proof vessels into the International Sanitary Convention. It did not become really general until just before the Second World War. Not only does rat-proofing eliminate R. rattus from vessels at sea, but its application to new buildings is also gradually eliminating the species from the large ports as they become modernized. At the same time, the resistant species R. norvegicus which is unaffected by modernization at the ports, is multiplying. The antagonism between the two species is helping to bring about the complete disappearance of R. rattus. The world-wide decline of the infection is commencing, one after the other, a number of foci, which appeared to be firmly established, are dying out. Bit by bit, plague is losing the ground it gained temporarily during the half-century of the modern pandemic, its latest and most spectacular losses being in India and Java.

#### THE FATE OF PLAGUE

Nevertheless, plague still clings to those positions where biological conditions have favoured its permanent establishment. Our present knowledge of these conditions enables us to identify most of the foci concerned and to measure their considerable extent during half a century, plague, by increasing the number of its permanent foci, has more than doubled its area of distribution, now on a world-wide scale as a result of territorial gains such as those in South Africa and the Americas.

However, it can safely be affirmed that the disease will never again have the opportunity it had at the beginning of the modern pandemic: it has been banished from the high seas and the great ports have been closed to it. On the seas, plague is now carried - as in the past - only in coastal waters, by small boats which are neither rat-proof nor deratted, and between ports still without facilities or supervision. Such ports, however, are still in the majority, and all over the world lighters, junks, sampans and coasters of all kinds remain rat-infested. oceanic plague has disappeared but maritime plague remains and certain areas offer it the same scope as it had in the past. It is safe to say that there will never again be a plague pandemic, it is certain, however, that, with its inherent epidemic tendencies, the disease will occasionally flare up in localized outbreaks and show that it is still a force to be reckoned with. While plague is at present quiescent, this must not blind us to the fact that the positions it holds are stronger than ever; entrenched within reach of all the strongholds of modern civilisation, it may well be a disease of the future.

Recent gains in knowledge permit us to have a better idea of this future. It seems certain that the introduction of murine infection into a plague-free area can only occur where R. rattus exists, and that the ensuing epizootic will be brief if R. rattus alone is present and can

only be prolonged if R. norvegicus is also found. It would seem certain that a focus can develop only where the infection can become established in wild or field rodents. This focus will be only temporary if too low a resistance and too high a mortality among these rodents prevent the infection from settling in one place and force it to keep moving into new areas in order to survive. On the other hand, inveterate foci will develop where sufficiently resistant species occur and a balance of infection is maintained between resistant and susceptible species.

## PROPHYLAXIS

Our better knowledge of the means whereby plague can spread should also make it possible to define control measures more clearly. In respect of quarantine, prophylaxis of the human infection remains unchanged, since the International Sanitary Regulation have wisely retained, through successive modifications, the provision specifying disinsection of patients and suspects as well as of their baggage, bedding and linen. Thus, the principle of preventing the interhuman transmission of bubo-septicaemic plague by human ectoparasites, whose importance has been shown above, has been safeguarded. Similarly, the prevention of murine infection by rat-proofing or traditional deratting, as defined in the International Sanitary Regulations, remains valid both for immediate and long-term prophylaxis.

Nonetheless, the WHO Expert Committee on Plague recognized that the Institut Pasteur de l'Iran was correct in pointing out the inaccuracy of the word "rodents" in certain definitions appearing in the International Sanitary Regulations. The Committee recommended its replacement by the word "rats", thus specifying that only those areas where rat plague is active (and not, for example, permanent plague foci among wild rodents without murine plague) should be declared "infected local areas" and treated as such from the quarantine standpoint.

For immediate national and long-term international prophylaxis, far-reaching changes in the accepted regulations are necessary. As regards immediate prophylaxis, there should be no deratting, which is a waste of time, labour and money; it should be replaced by the thorough disinsecting of houses and their occupants, thus breaking in one operation the chain of infection from rat to rat, from rat to man, and from man to man. There should be no vaccination, which is too slow and unreliable for immediate prophylaxis, but chemoprophylaxis by means of sulfonamides. There should be no cordon sanitaire or isolation measures, since these are rendered pointless by disinsecting, chemoprophylaxis of contacts, and treatment of patients with sulfonamides or antibiotics.

As regards long-term prophylaxis in infected territory, once it had been demonstrated that wild or field rodents alone play a part in the maintenance of propagation of the infection, it seemed logical to carry out eradication campaigns based on the destruction or at least disinsection of field rodents and their burrows. Because of certain prejudices arising

from previous failures, public health authorities were at first reluctant to undertake such campaigns, but the success of those carried out in certain inveterate foci in the USSR showed that they were the only means of obtaining lasting results.

Where long-term prophylaxis at the international level is concerned, certain definitions (as in the case of yellow fever) appear to be called for, i.e., the definition of "receptive areas" as those where R. rattus exists side by side with a sufficiently numerous and vulnerable field rodent fauna; of "critical areas" as inveterate wild foci together with the ports, even if free from infection, situated in their neighbourhood, and, finally, of "immune areas" as areas free from R. rattus, together with the neighbouring ports. These definitions might permit the immediate preparation of agreements for international assistance in the event of the invasion of a "receptive area" by plague. In this way the threatened country could be provided with everything necessary to ensure the rapid eradication of the disease before it had time to take root and spread.

An international programme for the detection and delimitation of "critical areas" should be drawn up, making it possible to designate "infective ports" and to plan the eradication of plague in the areas<sup>2/</sup> concerned. In the third report of the WHO Expert Committee on Plague<sup>2/</sup>, a programme of this type headed the Committee's "Recommendations for co-ordinated research."

#### REFERENCES

1. Pastukhov, B. N. "The Epizootic and Epidemic Situation in the Natural Foci of Plague in the USSR and Prophylactic Measures Taken," Bull World Health Org, 23 401-404, 1960.
2. Expert Committee on Plague Third Report, Technical Report Series No. 165, World Health Organization, Geneva, 1959.

## REVIEW OF PLAGUE

### STUDIES ON PLAGUE ANTIGENS

William D. Lawton, Ph.D.\*

Gel diffusion studies have revealed that Pasteurella pestis produces a great number of antigens -- 18 different ones have been identified in our laboratory and this represents only the minimum number. Many of these antigens are also produced by Pasteurella pseudotuberculosis (Table I). This represents a typical virulent and avirulent strain of P. pestis and an avirulent strain of P. pseudotuberculosis. Discussions of plague immunology usually revolve around just a few of these antigens, which I will review briefly.

F antigen, better known as Fraction I, is associated with the capsule. It is produced in abundance only at temperatures approaching 37° C and has been studied extensively by K. F. Meyer and co-workers, who have purified it and demonstrated that it is a protective antigen in several laboratory animals.

T antigen, the murine toxin, has been purified by Ajl and others and is the logical cause of plague deaths. Antitoxin by itself does not protect against infection, but may protect against toxemia after a bacteremia is established. The exact role that the toxin plays in the pathogenic process is still nebulous.

V and W antigens have been discovered and associated with virulence by Burrows and Bacon. I will have more to say about these two antigens later; for now, it is enough to note that Burrows considers that they are protective antigens.

The antigens associated with protection against plague then are F, V, W, and possibly T. It can be noted from Table I that avirulent strains of P. pseudotuberculosis do not produce any of these antigens, yet vaccination of guinea pigs with P. pseudotuberculosis establishes a solid immunity against plague (Table II). I would like to discuss our attempts to identify this protective factor.

This initial observation that live avirulent P. pseudotuberculosis can immunize against plague was merely a confirmation of much earlier reports by Rowland in 1912 and Schutze in 1932. Subsequent sonic oscillation of a P. pseudotuberculosis culture followed by centrifugation and sterilization by heating demonstrated that all the protective material was associated with the sonicated residue. The next step was assisted by a publication by Keppie, Cocking and Smith, stating that a nontoxic complex from P. pestis which immunized mice and guinea pigs could be

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TABLE I. ANTIGENS OF P. PESTIS AND P. PSEUDOTUBERCULOSIS.

ANTIGEN	<u>P. PESTIS</u>		<u>P. PSEUDOTUBERCULOSIS</u>
	ALEXANDER (VI)	A4 (AVI)	4C (AVI)
B	+	+	+
C	+	+	+
D	+	+	-
E	+	+	+
F	+	+	-
G	+	+	+
H	+	+	+
I	+	+	+
J	+	+	+
K	+	+	+
L	+	+	+
M	-	-	+
N	-	-	+
O	+	+	+
Q	+	+	+
T	+	+	-
V	+	-	-
W	+	-	-

TABLE II. PROTECTION AFFORDED BY P. PSEUDOTUBERCULOSIS AGAINST CHALLENGE WITH P. PESTIS.

VACCINE	DEAD/TOTAL
Live <u>P. pseudotuberculosis</u> Strain 4C	0/10
None	10/10

Guinea pigs challenged with 100 LD<sub>50</sub> dose of P. pestis (Alexander).

isolated by ultrasonic oscillation followed by solubilization of the residue in 0.05 M sodium bicarbonate buffer, pH 8.5. Treatment of P. pseudotuberculosis sonicated residue with bicarbonate buffer caused all of the protective activity to go into solution, making it quite probable that our protective factor and Keppie's immunizing complex contained the same antigen.

Several methods of purification were attempted. Figure 1 represents an attempt to fractionate the protective factor (PF) on DEAE cellulose. The crude material was poured through a cellulose column which was connected to a silica cell set up in a homemade recording spectrophotometer consisting of a monochromatic light source and a Brown recorder. A continuous reading of the optical density at 280 m $\mu$  was recorded, making it possible to test just the peaks for activity. As this figure shows, a great deal of the crude material did not absorb onto the cellulose, but came through in the wash. Elution with an increasing gradient of NaCl provided two more major peaks, but subsequent tests showed that all of the PF was associated with the wash.

Among several other fractionation methods tried, most of which failed to accomplish any significant purification, was a run in which we centrifuged the crude protective material in the Spinco Model L ultracentrifuge. This resulted in a dark precipitate that contained all of the PF. Since all of the soluble antigens remained in the supernate, a few washes in bicarbonate buffer followed each time with ultracentrifugation resulted in an opalescent product which we consider to be "purified" (Figure 2, the 27,000 x G for 30' indicates the amount of centrifugation; the fraction containing protective material is underlined).

When this purified PF was diffused in a gel plate against several complex plague antisera, no bands of precipitate were evident (Figure 3). Furthermore, injection of PF into a rabbit yielded antiserum that showed no bands against PF but one strong band against sonicated cell supernate. If anti-PF serum was absorbed with PF, this band was removed. The present hypothesis to explain these observations is that: (1) PF antigen is too large to diffuse in a gel plate, and (2) sonic oscillation splits a portion of the large PF molecule into a fragment that can diffuse into agar and react with anti-PF serum. Additional evidence for this hypothesis is that after treatment with sodium deoxycholate, purified PF will react in a gel plate with anti-PF serum. Sodium deoxycholate treatment also results in the liberation into the supernate of about 50% of the total protein in purified PF and in the destruction of the protective activity.

By diffusing anti-PF against several of the standard antigen-antibody systems described in the past, the antibody directed against PF was shown to be the same as the antibody directed against the lipopolysaccharide identified and purified by Davies a few years ago. He demonstrated that the purified lipopolysaccharide was not protective in animals.

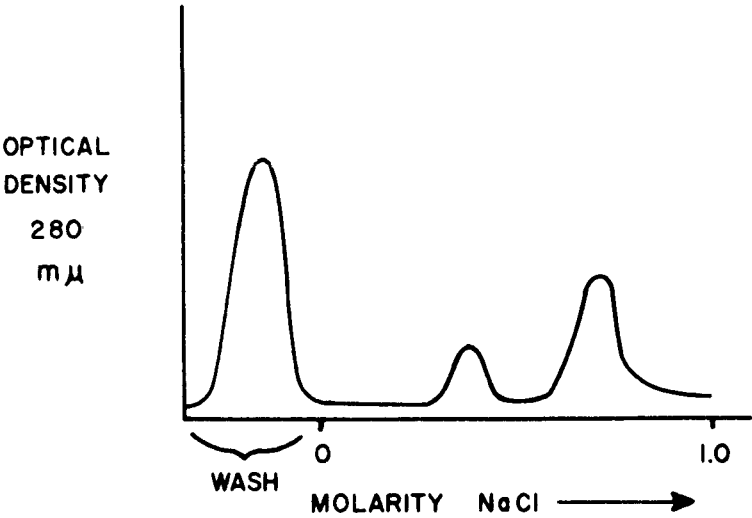


FIGURE 1. OPTICAL DENSITY OF PROTECTION FACTOR FRACTIONATED ON DEAE CELLULOSE

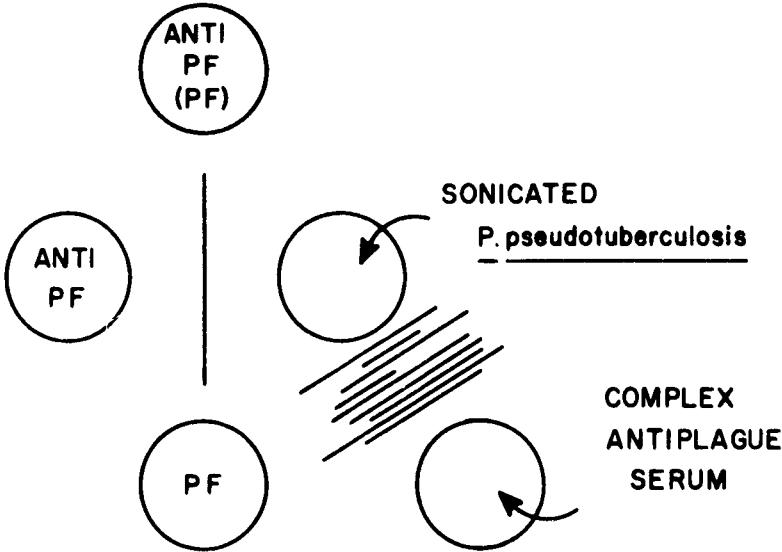
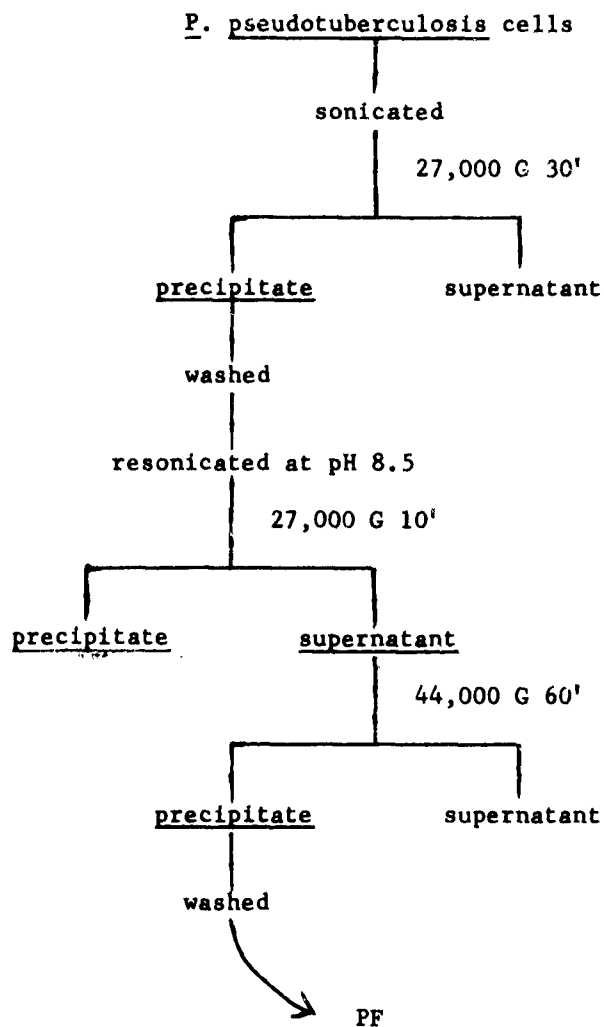


FIGURE 3. GEL DIFFUSION PATTERNS OF PROTECTIVE FACTOR AGAINST PLAGUE ANTISERA.

FIGURE 2. FLOW DIAGRAM OF A FRACTION METHOD FOR PROTECTION FACTOR.



Attempts to correlate immunity with a specific antibody obtained from immunized animals have not been successful. Anti-PF serum will not protect passively when injected into normal animals and several immunized guinea pigs have had no antibodies detectable by gel diffusion methods.

Another approach was taken that lends more support to the idea that classical antibody may not be involved in this type of immunity. The only strain of P. pestis that will not immunize guinea pigs when used as a live vaccine is strain TRU. If TRU did not produce the protective factor, then a comparison of purified PF from P. pseudotuberculosis and from TRU might indicate the essential ingredient of the protective factor. Identical procedures were used to prepare PF from each culture as previously mentioned. As predicted, the purified PF from the P. pseudotuberculosis culture protected guinea pigs but the PF from the TRU culture did not. Since neither PF showed any bands when diffused against complex antisera, an attempt was made to break down the molecule and to study the breakdown products by use of gel diffusion (Figure 4). One per cent sodium lauryl sulfate proved to be capable of changing the PF preparations so that at least seven different bands of precipitate reacted with antisera in gel plates. Enthusiasm for this approach ran high when it was shown that strain TRU completely lacked one of these antigens. Subsequent comparisons, however, demonstrated that certain nonprotective fractions from P. pseudotuberculosis cultures contained this antigen. Specifically, if in the process of preparing PF from P. pseudotuberculosis, distilled water is used in place of bicarbonate buffer, the protectivity is lost, but this nonprotective fraction still contains the same amount of this antigen that TRU lacks.

Although this method of breaking down a complex molecule with sodium lauryl sulfate shows some interesting information we have not been able to point to a specific band in a gel plate and say, "This antigen is essential for the complex to be immunogenic."

A recent development has shed further light on this type of immunity (Table III). In an attempt to determine the centrifugal force necessary to sediment PF, the crude material was centrifuged at 27,000 x G and the supernate spun at 105,000 x G. Unfortunately, this batch of crude material had been used for various tests during a 4-week period prior to this experiment and the immunogenic potency after 4 weeks storage at 5 C was less than it was originally (see 3-weeks results). This should have been 0 dead out of 8 total animals; but the interesting observation was that if the guinea pigs were challenged at 1 or 2 weeks postvaccination, the protection was better than at 3 weeks. Even the low speed material that looked as if it contained little or no protective factor at 2 or 3 weeks, looked good at 1 week. Table IV shows the results of challenging groups of guinea pigs at 1, 2 and 3 weeks after vaccination with 10 mg of PF prepared from different strains of P. pseudotuberculosis and P. pestis. Challenge 1 week after vaccination

TABLE III. PROTECTION AFFORDED GUINEA PIGS BY CENTRIFUGATES OF  
P. PSEUDOTUBERCULOSIS AGAINST CHALLENGE WITH VIRULENT  
P. PESTIS.

<u>P. PESTIS</u> (ALEXANDER) 100 LD <sub>50</sub> challenge after	VACCINE	
	27,000 x G (12 mg)	105,000 x G (6 mg)
	Dead/Total	Dead/Total
1 week	1/4	0/4
2 weeks	4/4	0/4
3 weeks	6/8	2/8

TABLE IV. PROTECTIVE INDEX\* IN GUINEA PIGS VACCINATED WITH  
 PROTECTIVE FACTOR FROM VARIOUS STRAINS AND  
 CHALLENGED WITH VIRULENT P. PESTIS.

STRAINS	PROTECTIVE INDEX POSTCHALLENGE		
	1 week	2 weeks	3 weeks
<u>P. pseudotuberculosis</u>			
4C	52	16	13
I	21	16	28
II	23	48	27
III	52	Inf	84
IV	Inf**	-	Inf
V	11	17	-
<u>P. pestis</u>			
D1	14	28	-
EV76	30	27	Inf
M23	-	Inf	Inf
14	-	19	32
Controls	9	7	8

\* Protective index equals  $\frac{\text{Number of dead animals}}{\text{Total number of animals}}$  times 100.

\*\* Infinity.

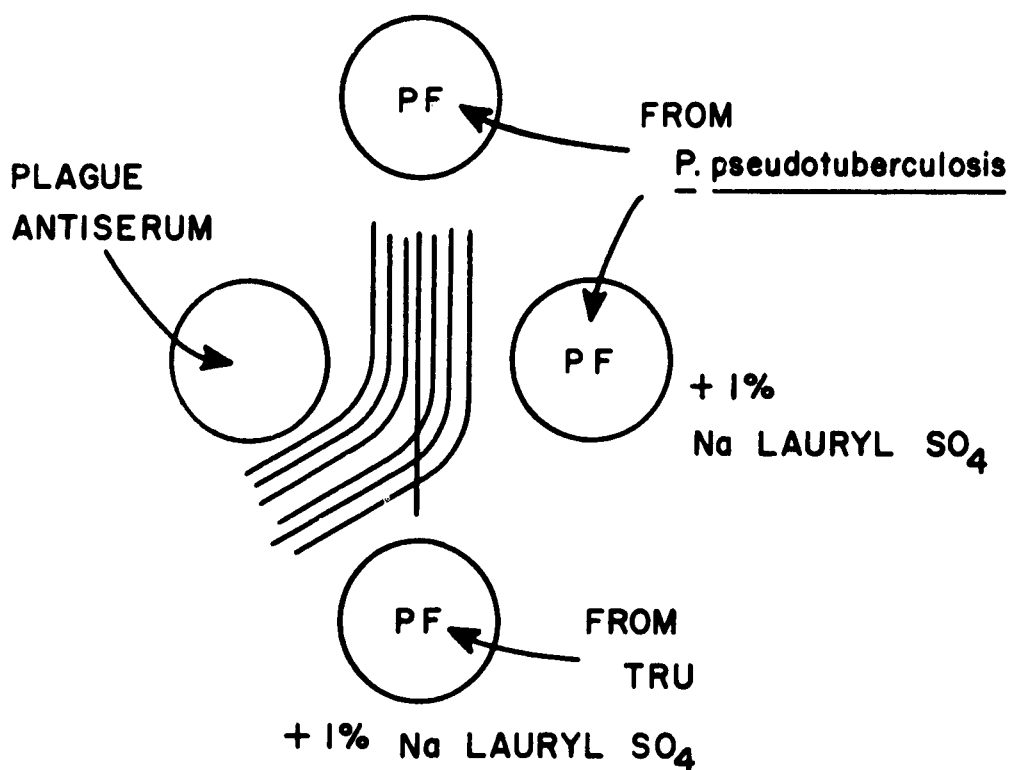


FIGURE 4. GEL DIFFUSION PATTERNS OF PROTECTIVE FACTOR FROM P. PESTIS (TRU) AND P. PSEUDOTUBERCULOSIS.

showed good protection in most cases. In view of the protection demonstrated as early as 1 week postvaccination, groups of 4 guinea pigs were challenged 1, 2, 3, 4, 5, 6 and 7 days after vaccination with 10 mg of PF prepared from P. pseudotuberculosis intravenously. All the vaccinated animals were very resistant to plague (Table V).

TABLE V. PROTECTIVE INDEX\* IN GUINEA PIGS CHALLENGED WITH P. PESTIS DURING THE FIRST WEEK AFTER IMMUNIZATION WITH PROTECTIVE FACTOR FROM P. PSEUDOTUBERCULOSIS.

DAY OF CHALLENGE Postvaccination	PROTECTIVE INDEX	
	Controls	Vaccinated
1	12	Inf**
2	12	23
3	12	40
4	14	Inf
5	12	Inf
6	-	Inf
7	9	Inf

\* Protective index equals  $\frac{\text{Number of dead animals}}{\text{Total number of animals}}$  times 100.

\*\* Infinity.

These data suggest that an endotoxin may be the cause of PF immunity. Although Davies has reported that efforts to obtain endotoxin from P. pestis by classical methods have failed to yield typical endotoxin, data obtained concerning PF show many similarities to results obtained with endotoxins, especially the association of a lipopolysaccharide-protein complex with PF, the immunity obtained only 1 day after vaccination, and the apparent lack of circulating specific antibody in immune animals indicating that some type of cellular immunity may be involved.

Of the many questions remaining to be answered on this problem, we are most actively concerning ourselves with how specific this type of immunity is; how can this immunity be measured, especially if the effects are cellular only; and what role does this protective factor play in the pathogenesis of plague?

I plan to devote the remaining time to a discussion of the two antigens that were produced by virulent, or potentially virulent, but not avirulent strains of *P. pestis*. V and W antigens were identified by Burrows and Bacon and, according to them, were correlated with resistance to phagocytosis in mice and necessary for the best live vaccine in mice. The biological effects associated with these antigens, however, have been observed by use of live strains that were either V+W+ or V-W-. Burrows observed that both antigens were always produced together and refers to them as the VW antigens. Our interest in trying to determine the role that each of these antigens played in the virulence of *P. pestis* prompted us to undertake the separation and purification of V and W (Figure 5).

The method we used to produce large batches of these antigens was to grow strain M23 at 26°C and inoculate the cells at a high level into 2 liter flasks each containing 300 ml of 5% Bacto Casitone and 0.04 M sodium gluconate and shake at 36°C overnight. In this manner, about 14 liters could be produced at one time. This procedure of growing the cells at 26°C and then incubating the cells at 36°C was designed to get around the fact that  $\text{Ca}^{++}$  is necessary for growth of virulent cells at 36°C but inhibitory for VW production. The cells were permitted to settle out, the supernate siphoned off and brought to 3.0 M with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitated proteins were dialysed against distilled water and treated in several different ways to separate V from W. Our purification is measured in terms of units of antigen per mg protein. A unit of antigen is defined as that amount which will just form a band of precipitate when diffused against a standard antiserum under standard conditions in a gel plate.

Time permits only a discussion of the best results (Figure 6) which shows the result of fractional precipitation with  $(\text{NH}_4)_2\text{SO}_4$ . The ordinate shows the units of antigen per mg protein. By taking a cut between 1.4 and 2.2 M  $(\text{NH}_4)_2\text{SO}_4$  a 20-fold purification was gained with about 90% recovery.

The 1.4-2.2 M fraction was dialysed and poured on a DEAE cellulose column. After washing the column with distilled water, 0.1, 0.3 and 0.5 M NaCl were successively passed through the column (Figure 7). The 0.1 M eluate contained most of the V antigen but no W, the 0.3 M eluate only traces of each, and the 0.5 M eluate W, but no V. A clean-cut separation of the two antigens was thus accomplished. A summary of the overall procedures is outlined in Figure 8. The best products contain 20 units of V per mg protein (about 100-fold purification) and 600 units of W per mg protein (about 1,000-fold purification). As judged by gel diffusion methods the V antigen preparation contained two other antigens, both in lower concentration than V, and the W antigen preparation contained a trace of one other antigen.

Other purification methods tried with results less encouraging than DEAE cellulose included continuous flow curtain electrophoresis, calcium phosphate chromatography, sephadex filtration, methanol fractionation and, acid and zinc precipitation.

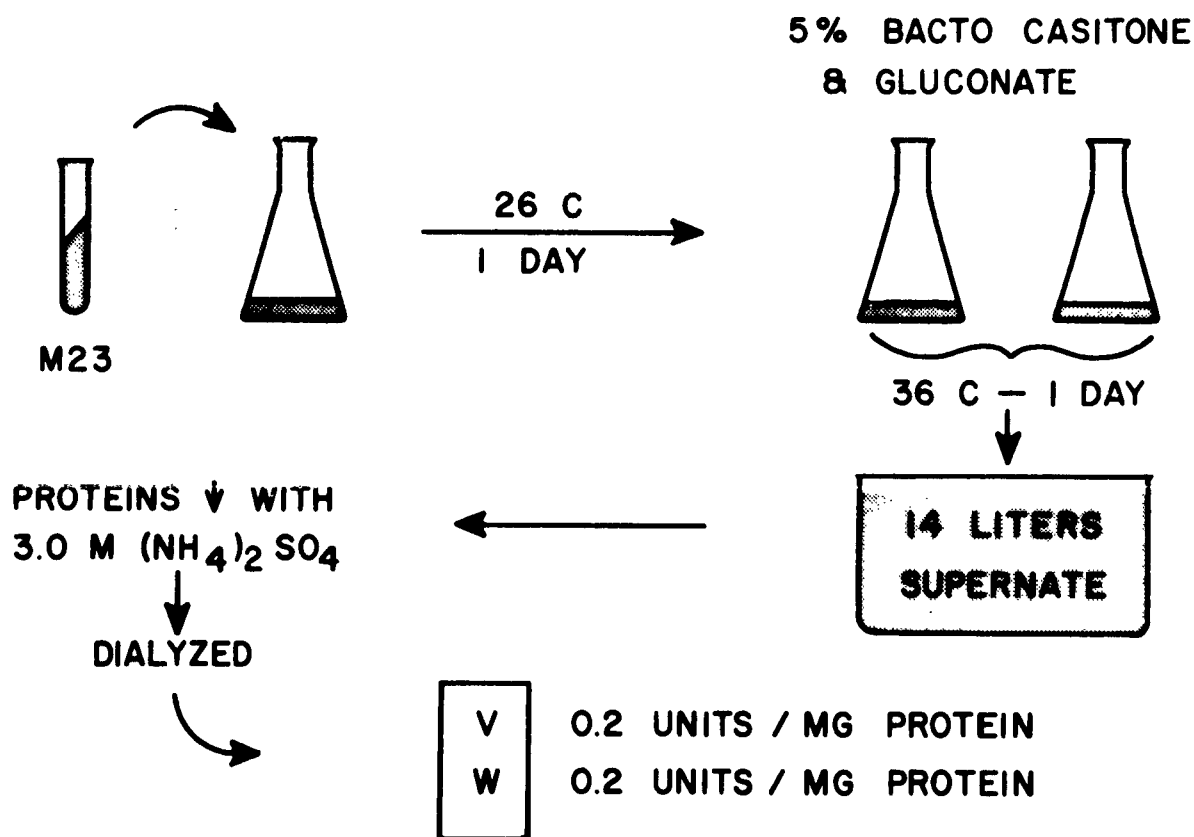


FIGURE 5. PROCEDURE FOR PRODUCING V & W ANTIGENS.

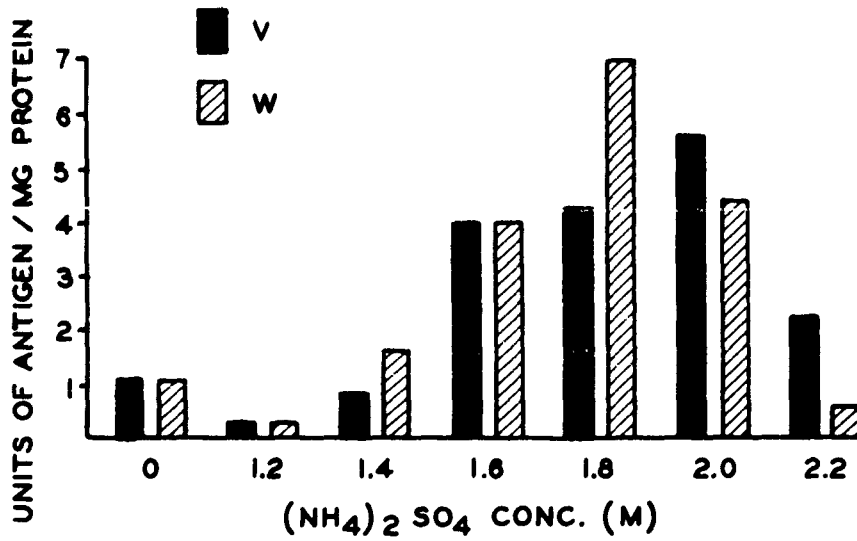


FIGURE 6. UNITS OF V AND W ANTIGEN IN FRACTIONALLY PRECIPITATED MATERIAL.

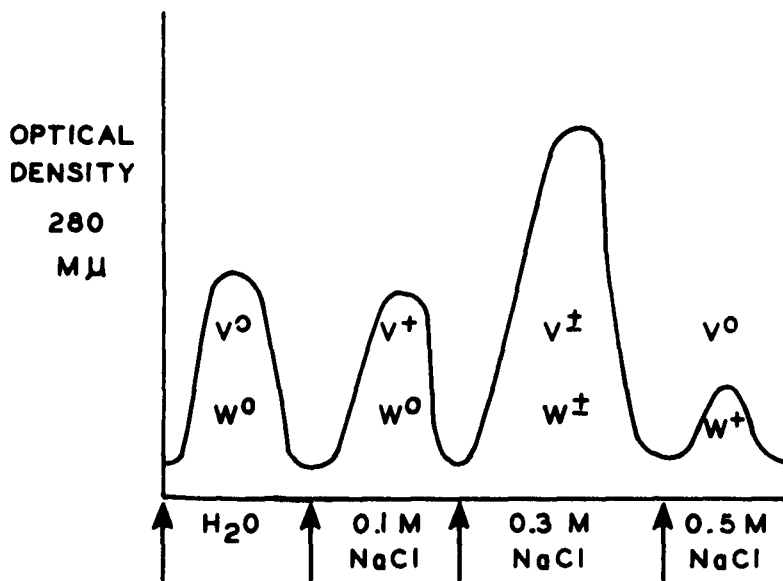
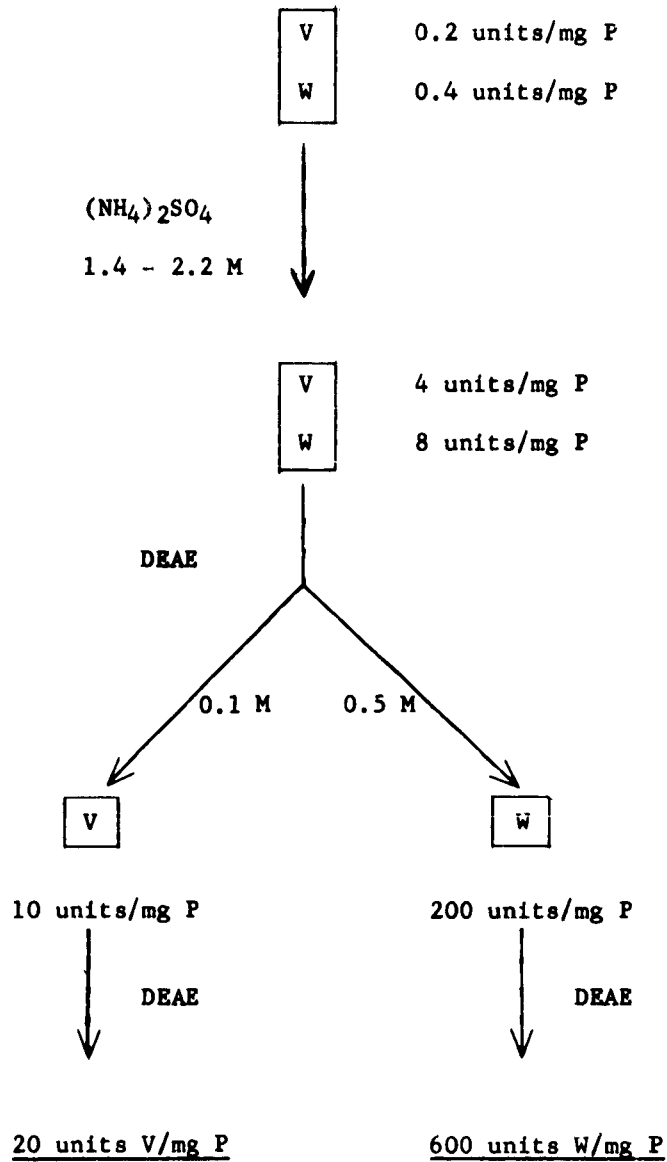


FIGURE 7. SEPARATION OF V & W ANTIGENS ON DEAE CELLULOSE.

FIGURE 8. FLOW DIAGRAM OF PROCEDURE FOR PRODUCING V AND W ANTIGENS.



Purification of these antigens has been hampered by their gradual instability (Figure 9). V antigen could be concentrated by pervaporation but W was destroyed. W antigen could be concentrated by dialysis against Carbowax (polyethelene glycol, without loss in titer but prolonged dialysis of W against cold distilled water resulted in the complete loss of W.

FIGURE 9. STABILITY OF V AND W ANTIGENS THROUGH PROCESSING.

<u>STAGE OF PROCESS</u>	<u>TOTAL UNITS OF ANTIGEN</u>	
	V	W
Supernate	8,300	66,500
↓		
5°C . 3 days	6,000	32,000
↓		
Dialysed	5,800	11,200
↓		
Lyophilized	3,300	7,600

Molecular weights, determined by the gel diffusion rate method of Polson, are shown in Table VI. The wide range for W leaves the accuracy of 160,000 in question.

TABLE VI. MOLECULAR WEIGHTS OF V AND W ANTIGENS.

<u>ANTIGEN</u>	<u>MOLECULAR WEIGHTS</u>	
	<u>Average</u>	
V	95,000	90,000
	85,000	
	90,000	
W	110,000	160,000
	225,000	
	145,000	

A few runs made with density gradient centrifugation have indicated that W antigen is lighter than other antigens of a lower molecular weight - thus suggesting that W antigen may be a lipoprotein.

Results of chemical analyses are still preliminary, but V appears on a dry weight basis to contain less than 1% nucleic acid and less than 1% carbohydrate with the rest being protein.

The results with W indicate the presence of less than 50% protein, more than 5% carbohydrate, and lipid. Analysis of W is still incomplete.

The use of purified V and W as vaccines in various animals has not been in progress long enough to draw definite conclusions. The impressions gained so far are that approximately 50 units (which is probably equivalent to about 50  $\mu$ g) of either V or W will elicit antibody response in rabbits. Antibody response in mice and guinea pigs has been poor so far.

By the use of anti-V and anti-W serum obtained in rabbits, we have been able to demonstrate passive protection of mice with anti-V but not with anti-W (Table VII); each column represents a separate run, so that two different anti-V sera and three different anti-W sera have been tested.

TABLE VII. PASSIVE PROTECTION AGAINST PLAGUE AFFORDED MICE BY ANTI-V AND ANTI-W RABBIT SERA.

RABBIT SERUM	MICE CHALLENGED INTRAPERITONEALLY WITH 100 LD <sub>50</sub> <u>P. PESTIS</u> (ALEXANDER)		
	DEAD/TOTAL		
Controls	10/10	10/10	10/10
Anti-V	0/10	-	0/5
Anti-W	-	10/10	5/5 5/5

## REVIEW OF PLAGUE

### STUDIES ON PLAGUE VACCINE

John E. Marshall, Major, MSC\*

During the past 6 months a series of pilot studies were conducted to screen various plague vaccines currently used. The first series consisted of a group of 7 nonviable vaccines tested against 5 challenge Pasteurella pestis strains, 195/P, DF 1, Miller, PKR 101, and PKR 159 with iron. The second series consisted of 3 nonviable vaccines, 4 living attenuated vaccines, and the survivors from 2 groups of mice inoculated subcutaneously with DF 1. This series was challenged with 195/P, DF 1, or Miller strains of P. pestis.

Challenge strains PKR 101 and PKR 159 with iron were eliminated since the results of the first experiment using these strains were essentially identical with the experiment using 195/P.

The test procedure was as follows: A group of 50 mice were vaccinated with 4 ED<sub>50</sub> doses of the test vaccine, prepared according to the recommendations of the laboratory of origin. The vaccine was given in 2 divided doses, 7 days apart. Thirty days following the second dose, the mice were subdivided at random into 5 subgroups of 10 animals each and challenged with a separate serial decimal dilution. As the number of infecting organisms in nature is unknown, we attempted to limit the number of organisms to subtoxic levels.

The method of scoring in brief involved assigning an arbitrary weighting coefficient to each 24-hour observation period. These daily observations were scored as follows: the over-all daily mortality was calculated and multiplied by the coefficient assigned; in these experiments a coefficient of 20 for day 1, 18 for day 2, 16 for day 3, etc., through day 10; thus EARLY deaths were emphasized. The challenges were given by the intraperitoneal route and in nearly all cases the tests were over by day 10.

The Porton vaccine was toxic to white mice in a dilution of 1:50 used in these experiments. The animals developed lesions at the site of inoculation which healed prior to challenge. No lesions were observed in mice immunized with any other vaccine. Mice inoculated with living P. pestis strain EV were mildly ill for 24 hours, the fur was ruffled and their water intake was diminished. They appeared normal at 48 hours.

With the exception of mice infected with DF1, no deaths occurred during the immunization period. The mortality rates of mice inoculated

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subcutaneously with  $10^6$  viable P. pestis (DF 1 were 52% for mice inoculated with a 37 C grown culture and 46% for those inoculated with a 25 C culture. Survivors had swollen lymph nodes at the site of inoculation at the time of challenge 28 days later.

Figure 1 shows the results of a challenge experiment. The number of viable organisms in each series was determined by duplicate plate counts at the time of challenge. In the control groups, 3 additional dilutions were included to permit the calculation of the  $LD_{50}$  for each challenge strain. All challenge strains were grown in veal infusion broth for 20 hours at 37 C, centrifuged at 4 C for 15 minutes and resuspended in saline.

Figure 2 shows the results of experiments using strain 195/P for challenge. Immunization with 0.1% formalin-killed P. pestis DF 1 in saline or Bayol-Arlacel provided no protection against challenge with P. pestis 195/P. The protection afforded the survivors of living DF 1 challenge experiments is probably due to the small amount of Fraction I liberated during the infection. Protection was afforded by all of the recommended plague vaccines and by purified Fraction I. The addition of Fraction I to the killed DF 1 vaccine protected mice to a similar degree as the Fraction I alone.

All living attenuated strains of P. pestis used in these experiments afforded good protection against virulent 195/P challenge. Lack of antigens V W in strain 1122 or P in EV 76 and PKR 159 did not appear to render these strains less effective as immunizing agents.

Figure 3 shows the results of the experiments using DF 1 as the challenge organism. For comparison with the 195/P series the scores have been multiplied by a factor 1.39. The nonviable vaccines offered little or no protection to mice challenged with DF 1. It is interesting to note that the killed homologous vaccines were ineffective. The living attenuated vaccines were somewhat more effective while survival from infection with DF 1 conferred a high degree of immunity to a second attack.

Figure 4 shows the results of experiments using the Miller strain of P. pestis as the challenge organism. For sake of direct comparison, all scores obtained in the test were multiplied by a factor 1.09. The results are similar to those for 195/P though immunization with Fraction I does not appear as solid in this instance. Killed DF 1 vaccine did not confer protection against infection with a classical strain of P. pestis.

The addition of Fraction I to the killed DF 1 vaccine while increasing the protective power of the vaccine did not approach the value of the Fraction I alone.

# METHOD OF DETERMINING PROTECTION SCORE FOR EVALUATING PLAGUE VACCINES

NUMBER OF ORGANISMS	DEATHS IN 10 MICE BY DAYS									
	1	2	3	4	5	6	7	8	9	10
7,480,000	5	4	0	1						
748,000	0	3	6	1						
74,800	0	0	7	2	1					
7,480	0	0	9	1						
748	0	0	6	3	1					
FACTOR	20	18	16	14	12	10	8	6	4	2

PROTECTION SCORE = 1000 - (NO MICE DYING/DAY X FACTOR)

FIGURE 1.

# COMPARATIVE PROTECTION OF PLAGUE VACCINES

CHALLENGE 25-250,000 LD<sub>50</sub> IP P. PESTIS (195/P)

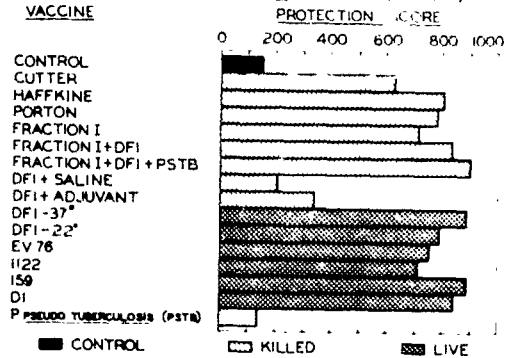


FIGURE 2.

# COMPARATIVE PROTECTION OF PLAGUE VACCINES

CHALLENGE 8-80,000 LD<sub>50</sub> IP P. PESTIS (DFI)

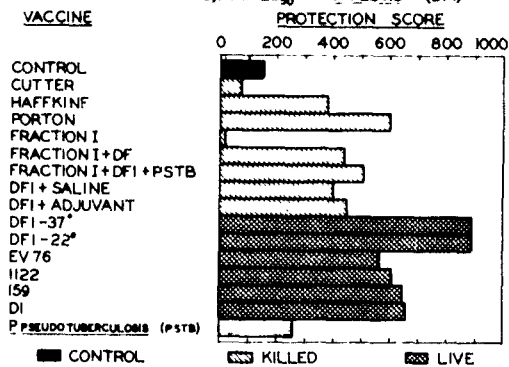


FIGURE 3.

# COMPARATIVE PROTECTION OF PLAGUE VACCINES

CHALLENGE 18-180,000 LD<sub>50</sub> IP P. PESTIS (MILLER)

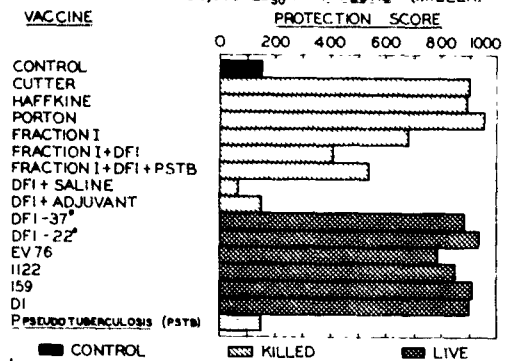


FIGURE 4.

Good protection was afforded by immunization with Cutter, Haffkine, and Porton vaccines.

Prior experience with living attenuated P. pestis rendered the mice immune to infection with the Miller strain. The lack of the P or V W factors in living attenuated vaccines did not adversely effect their protective properties.

The data are too limited to draw any firm conclusions at this time. A wider selection of challenge strains must be studied to determine variation in response to immunity conferred by different plague vaccines. Ultimately the respiratory route of challenge will be used to determine the effectiveness of the test vaccines against respiratory plague.

## REVIEW OF PLAGUE

### THE RELATIONSHIP BETWEEN THE PLAGUE BACILLUS AND THE PHAGOCYTTIC DEFENSE SYSTEM OF THE HOST

Werner A. Janssen<sup>\*</sup>

After the discovery of the etiologic agent of plague, the earliest workers in the field observed that the exudates from plague victims were teeming with bacilli and leukocytes, yet few, if any, of the phagocytic cells contained bacilli. Figure 1 is an impression smear of the spleen of a guinea pig that died of plague. This ability of Pasteurella pestis to resist phagocytosis has long been thought to be the major determinant of its virulence.

The phagocytosis resistance of P. pestis grown in vivo can be demonstrated in standardized test systems in vitro. Phagocytes from blood or exudates are mixed with a known ratio of bacilli and incubated under carefully controlled conditions. As you can see in Figure 2 there is no evidence of phagocytosis.

When P. pestis is grown in vitro at 26 C or less the organisms are highly susceptible to phagocytosis (Figure 3).

As shown in Table I, the relative resistance or susceptibility of a virulent strain can be measured in the in vitro test system by counting the number of organisms ingested by 100 neutrophils and 100 macrophages and/or determining the percentage of these phagocytes that contain bacilli. Either index shows the striking difference in phagocytosis resistance between cultures grown in vivo and in vitro.

Many workers have contributed circumstantial evidence in favor of the hypothesis that phagocytosis resistance is the major virulence determinant of P. pestis. I would like to review, very briefly, some of the evidence that we have contributed. The data presented in Figure 4 show that when virulent P. pestis grown in vivo were injected intratracheally into guinea pigs the number of viable organisms recovered from the lungs at various time intervals continued to increase from the time of injection, and there was relatively little indication of phagocytosis by leukocytes observed in the impression smears of the lungs; whereas, injection of the same strain grown in vitro at 26 C was followed by a rapid decrease in viable organisms in the lungs during the first 16 hours, and many of the organisms were taken up by phagocytes.

When the fate of phagocytosis-resistant and -susceptible organisms was followed after intracardial injection into guinea pigs, a rather puzzling observation was made. Both types of P. pestis disappeared from the blood at the same rate, however, the resistant type increased in number much earlier than the susceptible type (Figure 5).

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FIGURE 1. IMPRESSION SMEAR OF SPLEEN OF GUINEA  
PIG DEAD OF PLAGUE, SHOWING P. PESTIS  
AND LEUKOCYTES.



FIGURE 2. IN VITRO PHAGOCYTOSIS.

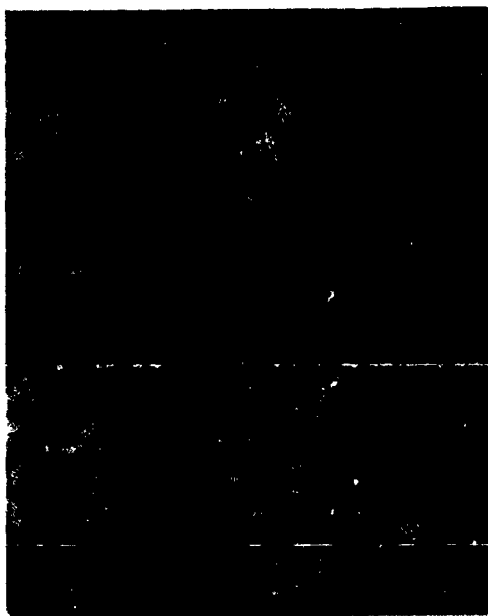


FIGURE 3. DEMONSTRATION OF IN VITRO PHAGOCYTOSIS  
SUSCEPTIBILITY. (P. PESTIS GROWN AT 26 C)

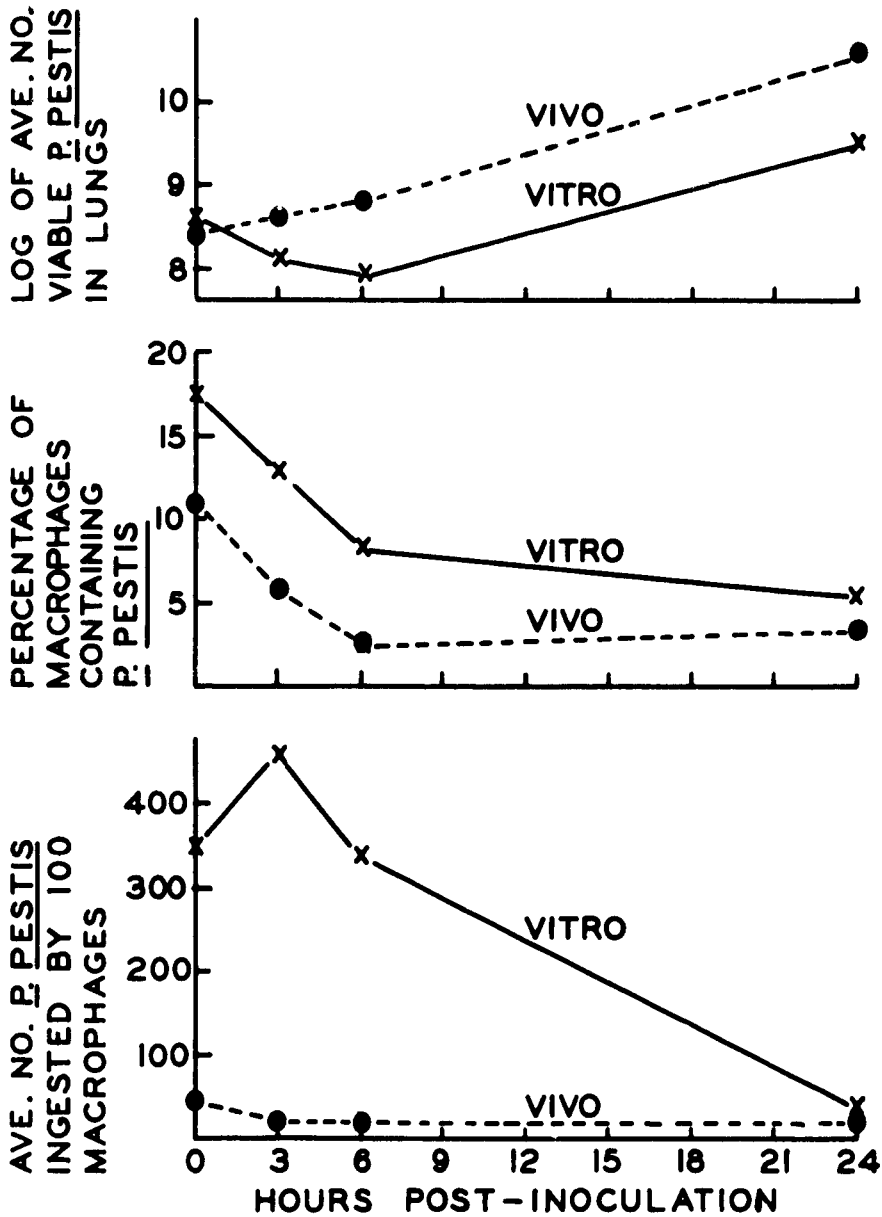


FIGURE 4. CORRELATION BETWEEN THE LUNG CLEARANCE PHENOMENON AND PHAGOCYTIC ACTIVITY OF MACROPHAGES IN GUINEA PIG LUNGS.

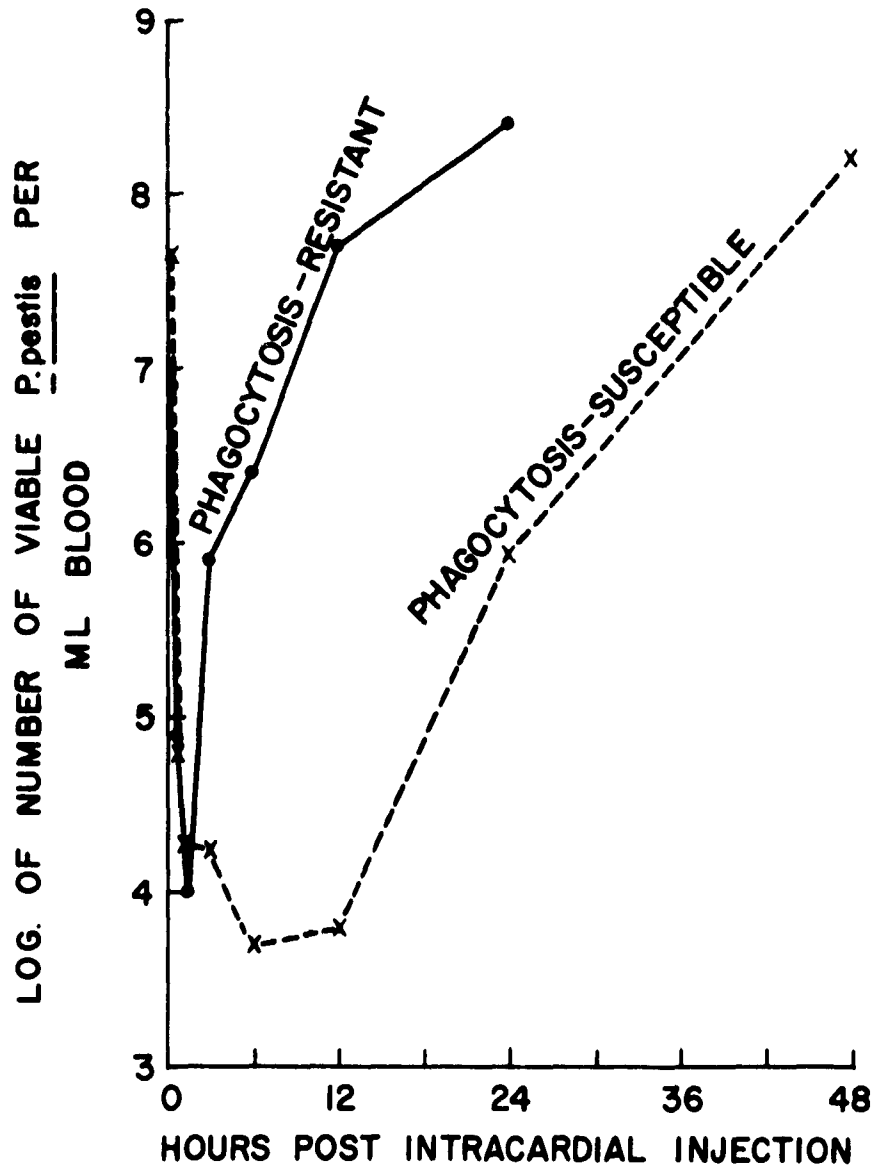


FIGURE 5. P. PESTIS IN GUINEA PIG BLOOD.

TABLE I. A COMPARISON OF THE PHAGOCYTOSIS RESISTANCE OF P. PESTIS  
(ALEXANDER STRAIN) GROWN IN VITRO AND IN VIVO.

GROWTH CONDITION	LEUKOCYTE SOURCE GUINEA PIG NO.	BACTERIA/ LEUKOCYTE	PHAGOCYTIC ACTIVITY <sup>c</sup>	
			NEUTROPHILE	MACROPHAGE
<u>In vitro</u> <sup>a/</sup>	1	19	34/27	268/78
<u>In vivo</u> <sup>b/</sup>	1	21	1/1	10/7
<u>In vitro</u>	2	14	45/30	256/74
<u>In vivo</u>	2	32	3/3	6/4
<u>In vitro</u>	3	16	70/42	512/86
<u>In vivo</u>	3	15	13/11	94/38

a. Grown in Difco heart infusion broth at 26 C.

b. Grown in the peritoneal cavity of guinea pigs.

c. Numerator - Number of intracellular bacteria in 100 neutrophils  
or macrophages.

Denominator - Percentage of neutrophils or macrophages containing  
bacteria.

Assay of the number of viable organisms in the liver, spleen, lungs and kidneys revealed that most of the organisms disappearing from the blood were taken up in the liver and spleen (Figure 6). The phagocytosis-susceptible type were apparently initially killed off in large numbers, while the resistant type were able to increase continually from the start.

As may be seen in Figure 7 histologic sections of the organs showed that the so-called phagocytosis-resistant type were taken up by the fixed macrophages of the reticulo-endothelial system of the liver and spleen just as the susceptible types were. The fixed phagocytes in the lungs and kidneys do not have access to bacteria in the blood, so these organs had little blood filtering effect.

Sections of the lungs following intratracheal injection of P. pestis revealed essentially the same thing (Figure 8). Fixed alveolar macrophages often contained large numbers of the resistant as well as phagocytosis-susceptible types. This indicated that the ability of P. pestis to resist phagocytosis was limited to the system of free phagocytes in the host, and did not apply to the fixed phagocyte system.

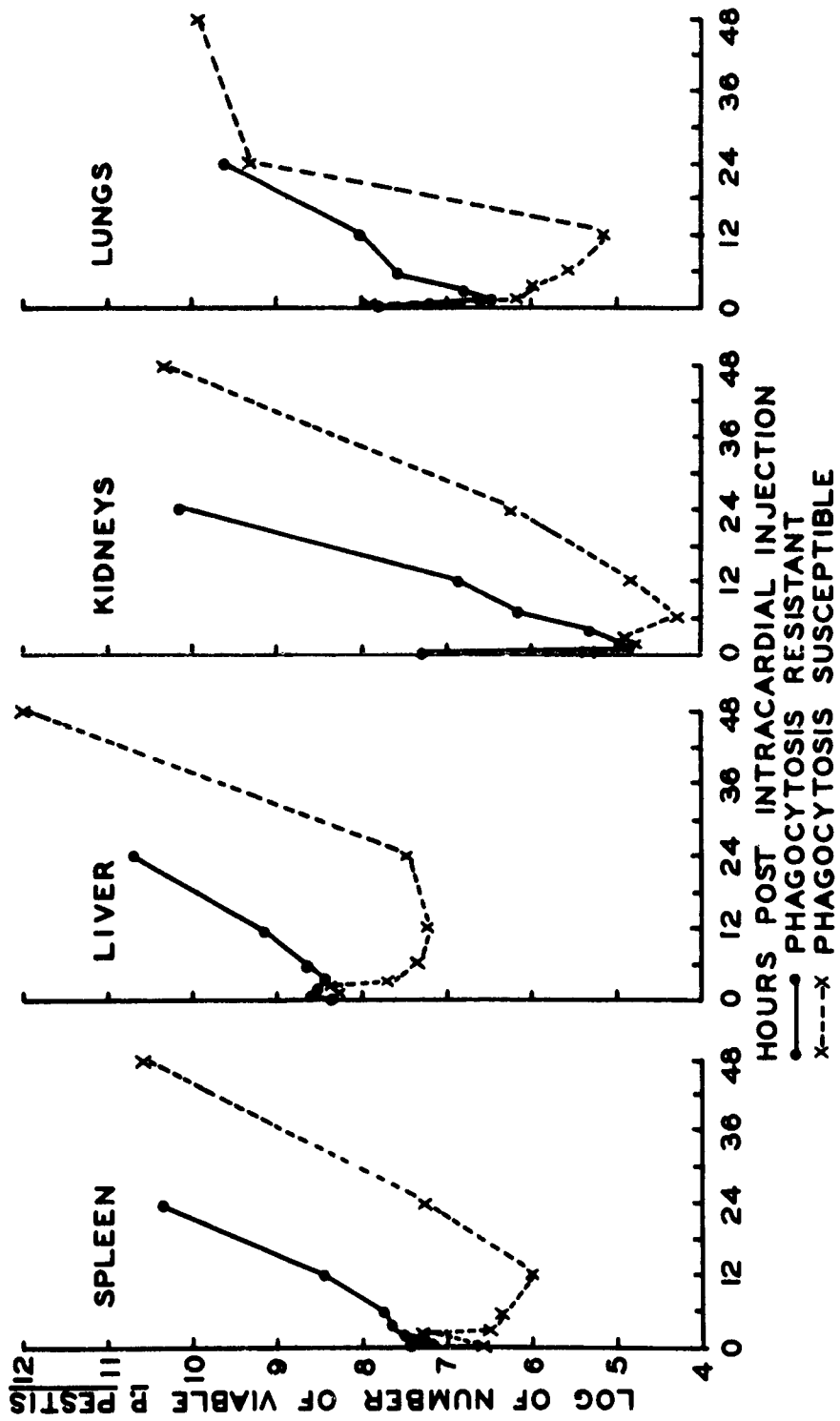


FIGURE 6. NUMBER OF VIABLE P. PESTIS IN GUINEA PIG ORGANS



FIGURE 7. LIVER SECTION SHOWING KUPFFER CELLS  
CONTAINING P. PESTIS. (Giemsa)



FIGURE 8. LUNG SECTION SHOWING ALVEOLAR  
MACROPHAGES. (Giemsa)

Burrows et al, and Cavanaugh and Randall have presented evidence that virulent P. pestis when ingested by neutrophils, are subsequently killed, but that the organisms multiply within macrophages. These experiments involved systems in which phagocytes were permitted to ingest susceptible P. pestis and any uningested bacteria were killed by antibiotics in the media surrounding the phagocytes in vitro. The phagocytes were either observed directly or disrupted by various means, and the number of organisms remaining after various intervals was determined by direct and/or viable counts. We repeated this type of study but instead of killing the extra-cellular bacilli with antibiotics, we attempted to wash away the uningested organisms by several washing cycles followed by light centrifugation. Control studies indicated that this was very effective. Our results tended to confirm the results of these other workers. However, in our studies, as in theirs, direct evidence of killing was based on morphologic evidence of degeneration of the intracellular bacteria, and assays of viability following rather rigorous mechanical or chemical disruption of the leukocytes (Table II).

Morphologic evidence of killing included in large part the change from the bacillary form to a spherical form, as seen in this neutrophil in Figure 9. Direct observation of these "spheroplasts" within neutrophils for 24 hours failed to detect any further degenerative or necrotic changes in the bacilli.

We were able to show that both virulent and avirulent P. pestis can readily be converted into spheroplast-like forms by egg white lysozyme (Figure 10, Table III). These "spheroplasts" are osmotically stable, viable, and retain the surface antigens which render them resistant to phagocytosis. Since the lysozyme in leukocytes is thought to be an important enzyme involved in the destruction of bacteria following phagocytosis, we suggest that the morphologic changes in P. pestis occasionally observed in phagocytes may be due to lysozyme, but these changes do not necessarily indicate that the bacteria are dead. It is conceivable that the weakened cell walls of the ingested bacteria may render them more susceptible to lethal damage during mechanical or chemical disruption of the phagocytes in the various test procedures which I have mentioned. This might give a false impression that the phagocytes were killing the bacteria.

More recent circumstantial evidence from our laboratory argues strongly against the hypothesis that the ability of P. pestis to resist phagocytosis is the major determinant of its virulence.

Fukui found that when virulent P. pestis organisms were grown at 5 C they were attenuated, but rapidly regained virulence upon incubation at 37 C for as little as 6 hours. There was no concurrent increase in phagocytosis resistance associated with this recovery of virulence for guinea pigs (Figure 11).

When the fully toxigenic avirulent strain A-4, and the virulent non-encapsulated strain M-23 were grown under conditions which induced phagocytosis resistance in the virulent strain Alexander, the avirulent strain also became resistant while the virulent non-encapsulated strain did not (Table IV).

TABLE II. THE EFFECT OF PHAGOCYTOSIS BY GUINEA PIG LEUKOCYTES ON P. PESTIS IN AN IN VITRO SYSTEM.

LENGTH OF INCUBATION IN HOURS <sup>a</sup> /	NO. VIABLE <u>P. PESTIS</u> PER ML OF SYSTEM	NO. INTRACELLULAR <u>P. PESTIS</u> PER 100		% PHAGOCYTES CONTAINING <u>P. PESTIS</u>	
		NEUTROPHILES	MACROPHAGES	NEUTROPHILES	MACROPHAGES
	$\times 10^7$				
0	3.8	468	1654	85	100
1	2.6	284	1504	69	100
3	1.7	196	1144	54	98

a. Mixture of phagocytes and bacteria was incubated 15 minutes, then extra-cellular bacteria were removed by washing before first sample was taken.

TABLE III. EFFECT OF LYSOZYME ON VIRULENT P. PESTIS.

CULTURE GROWN	LYSOZYME TREATMENT	% "PROTOPLASTS" IN CULTURE	VIABLE ORGANISMS $\times 10^9$ /ml	PHAGOCYTTIC INDEX <sup>a</sup> /	
				NEUTROPHILE	MACROPHAGE
<u>In vivo</u>	-	0	2.3	4/4	4/4
<u>In vivo</u>	+	67	2.2	2/2	4/4
<u>In vitro</u> 26 C	-	0	1.8	551/88	978/100
<u>In vitro</u> 26 C	+	2	1.4	545/81	1051/100

a. Numerator - Number organisms ingested by 100 phagocytes.

Denominator - % phagocytes containing organisms.

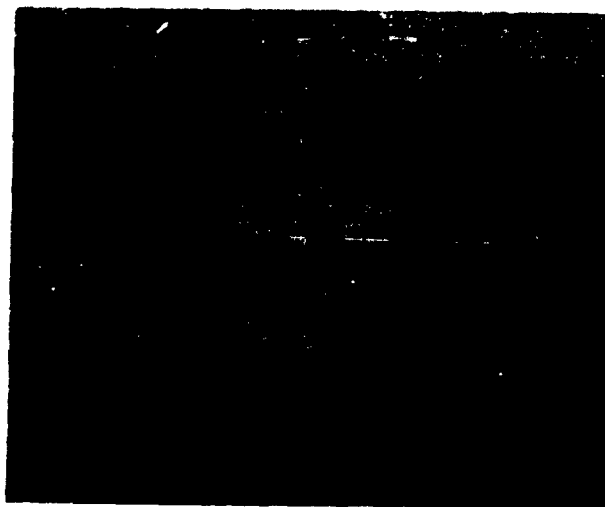


FIGURE 9. GUINEA PIG NEUTROPHILE CONTAINING  
"SPHEROPLAST-LIKE" FORMS OF P. PESTIS.  
(Giemsa)

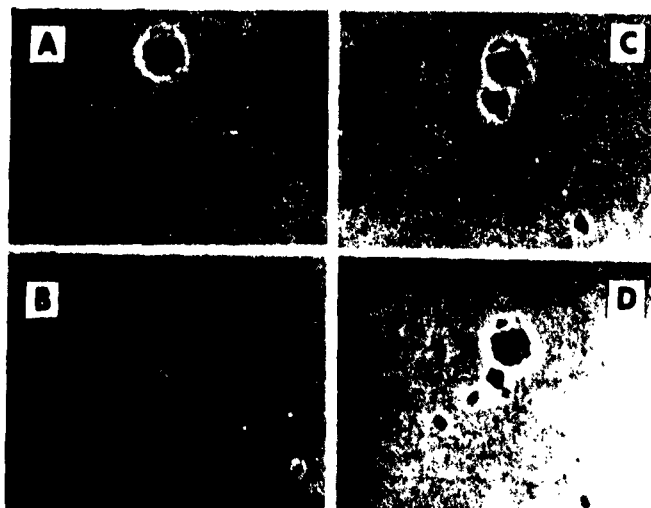


FIGURE 10. GROWTH OF "SPHEROPLAST-LIKE" FORMS  
OF P. PESTIS. (TIME LAPSE WITH PHASE  
CONTRAST ILLUMINATION)

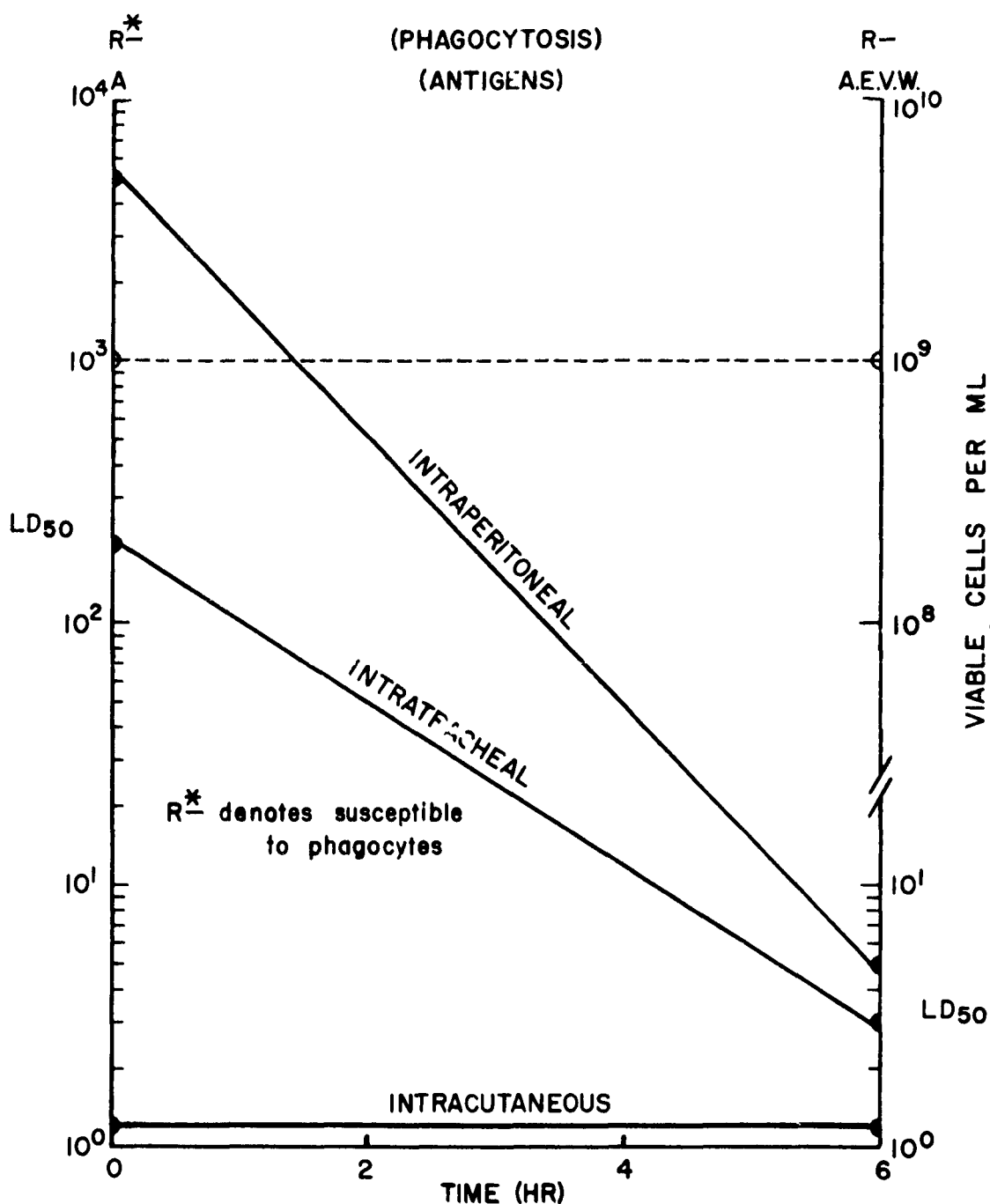


FIGURE II. ENHANCEMENT OF VIRULENCE FOR GUINEA PIGS IN NON-MULTIPLYING CELLS.

TABLE IV. A COMPARISON OF THE PHAGOCYTOSIS RESISTANCE OF A NUMBER OF P. PESTIS STRAINS.

<u>P. PESTIS</u> <u>STRAIN</u>	MEDIUM	INCUBATION TEMPERATURE	<u>BACTERIA</u> <u>LEUKOCYTE</u>	<u>PHAGOCYTIC ACTIVITY</u> <sup>a/</sup>		DEGREE OF RESISTANCE
				<u>NEUTROPHILE</u>	<u>MACROPHAGE</u>	
Sterile Control	HIB <sup>b/</sup>	--	0.0	0/0	0/0	--
Alexander	HIB	26 C	6.8	90/35	373/77	0
Alexander	HIB + 5% blood	37 C	6.9	38/25	92/44	+++
A-4 Avirulent	HIB + 5% blood	37 C	5.8	32/26	64/40	+++
A-12 Avirulent	HIB + 5% blood	37 C	9.7	194/54	444/82	0
M-23	HIB + 5% blood	37 C	9.2	95/51	252/84	0
M-23	<u>In vivo</u> <sup>c/</sup>	> 37 C	14.5	96/67	897/100	0
Alexander	<u>In vivo</u>	> 37 C	18.8	10/8	139/58	++++

a. Numerator - Number of intracellular bacteria in 100 neutrophils or macrophages.

Denominator - Percentage of neutrophils or macrophages containing bacteria.

b. HIB - Difco heart infusion broth.

c. In vivo - Grown in the peritoneal cavity of a guinea pig.

The degree of virulence of these strains did not change however. It is also interesting to note that toxigenic avirulent strains grow equally as well as virulent strains in guinea pig blood maintained under simulated in vivo conditions of temperature and oxygenation. This led us to suspect that the major determinant of P. pestis' virulence is its ability to survive and multiply within the free and fixed phagocytic systems of the host, and I would like to present our evidence in support of this hypothesis.

When the intraperitoneal LD<sub>50</sub> of virulent phagocytosis-susceptible P. pestis in normal guinea pigs was compared with the dose in guinea pigs which had a pre-existing casein induced peritoneal exudate, no significant difference was noted (Table V). Control studies showed that the ratio of phagocytic cells to bacteria

TABLE V. THE EFFECT OF PREMOBILIZED PHAGOCYTES ON THE INTRAPERITONEAL LD<sub>50</sub> OF VIRULENT P. PESTIS IN GUINEA PIGS.

EXPERIMENT	UNTREATED CONTROL GUINEA PIGS		GUINEA PIGS WITH INDUCED PERITONEAL EXUDATE <sup>a/</sup>	
	LD <sub>50</sub>	95% CONFIDENCE LIMITS	LD <sub>50</sub>	95% CONFIDENCE LIMITS
1	9.2	4.8 - 17.5	7.2	4.2 - 12.2
2	42.0	22.0 - 79.0	19.0	7.2 - 47.5
3	19.0	9.5 - 38.0	15.0	4.5 - 49.5

a. Peritoneal exudate induced by intraperitoneal injection of 25 ml of 7.6% sodium caseinate solution 24 hr preceeding challenge.

Note: Method Litchfield and Wilcoxon used to calculate LD<sub>50</sub> and confidence limits.

in the peritoneal exudates, was approximately 40,000,000 to 1, so that the chance of the bacteria escaping phagocytosis must have been very small. Therefore, it appeared that phagocytosis had no protective effect.

In these experiments guinea pig phagocytes were incubated with virulent P. pestis in vitro for 6 hours, the uningested organisms were removed by washing, and the number of ingested cells was determined by direct and viable counting methods. Comparative LD<sub>50</sub> titrations in mice were carried out using dilutions of suspensions of leukocytes containing a known number of P. pestis, and using dilutions of a sample of the same culture used in the test system, but which had not been so treated. The LD<sub>50</sub> of P. pestis contained within free phagocytes from guinea pigs was not significantly different than the LD<sub>50</sub> of nonphagocytosed organisms, even though the bacteria may have been within the phagocytes for as long as 6 hours. It should be noted that in the closed in vitro systems used to study phagocytosis, as a rule the maximum number of bacteria will be taken up within 30 minutes. So again, it appeared that ingestion of P. pestis by guinea pig phagocytes had no effect on their viability or virulence (Table VI).

Since previous experiments revealed that P. pestis, like most bacteria, is removed from the blood by the reticulo-endothelial system, and that large numbers of the phagocytosis-susceptible type are apparently killed, it seemed logical that an LD<sub>50</sub> by the intracardial route should be relatively high especially when compared to an LD<sub>50</sub> by the intradermal route. A comparison of the LD<sub>50</sub>'s of phagocytosis-susceptible P. pestis injected into guinea pigs were not significantly different (Table VII). This indicated that the reticulo-endothelial system is not an effective defense against plague either, at least in guinea pigs.

TABLE VI. COMPARATIVE LD<sub>50</sub> IN MICE OF NONPHAGOCYTOSED P. PESTIS AND P. PESTIS CONTAINED WITHIN GUINEA PIG PHAGOCYTES.

EXPERIMENT NUMBER	LD <sub>50</sub> OF NONPHAGOCYTOSED <u>P. PESTIS</u>		LD <sub>50</sub> OF PHAGOCYTOSED <u>P. PESTIS</u>	
	VIABLE COUNT	DIRECT COUNT	VIABLE COUNT	DIRECT COUNT
1	23 (15.5-34)	110 (73-165)	15.4 (7.3-31.3)	42 (20-88.2)
2	7.2 (2.4-21.2)		35 (11.2-108.5)	110 (29-407)

Note: Method of Litchfield and Wilcoxon used to calculate LD<sub>50</sub>. 95% confidence limits shown in parentheses.

TABLE VII. COMPARATIVE LD<sub>50</sub> OF P. PESTIS FOR GUINEA PIGS WHEN INJECTED INTRACARDIALLY AND INTRADERMALLY.

EXPERIMENT NUMBER	INTRACARDIAL ROUTE		INTRADERMAL ROUTE	
	LD <sub>50</sub>	CONFIDENCE LIMITS	LD <sub>50</sub>	CONFIDENCE LIMITS
1	20.0	10 - 40	9.4	3 - 26
2	20.0	10 - 38	14.0	9 - 38
3	5.4	3.4 - 8.5	2.4	0.8 - 6.7

Note: Method of Litchfield and Wilcoxon used to calculate LD<sub>50</sub> and 95% confidence limits.

Direct evidence that phagocytosis is not effective in killing virulent P. pestis has also been obtained. Time lapse motion pictures have been taken in which a neutrophile was observed to ingest a number of P. pestis cells; no change in the morphology of the bacteria resulted during a 2-hour period of continuous observation under the phase microscope. These movies are available to any interested skeptics. Figure 12 represents a typical example of studies in which guinea pig phagocytes were observed to ingest P. pestis; after permitting varying lengths of intracellular residence the bacteria were released by lysing the phagocyte. As you can see, the bacteria were able to multiply. This was true whether the phagocyte was a neutrophile or a macrophage. We intend to exploit this technique further in order to study the fate of avirulent as well as virulent P. pestis in phagocytes.



FIGURE 12. ACTIVE GROWTH OF INTRACELLULAR  
P. PESTIS RELEASED BY ELECTRICAL  
LYSIS OF GUINEA PIG NEUTROPHILE.  
(TIME LAPSE WITH PHASE CONTRAST  
ILLUMINATION)

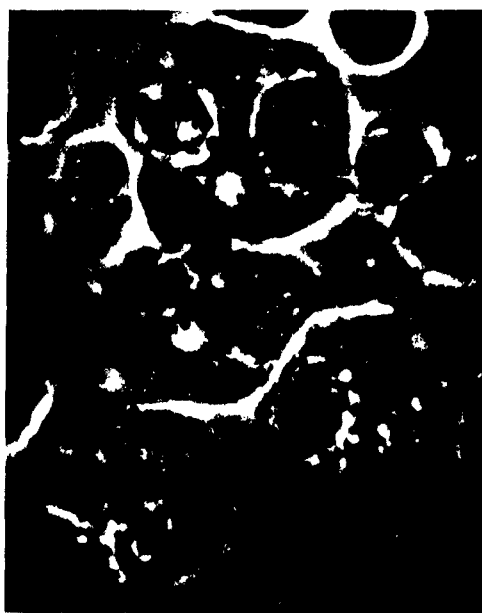


FIGURE 13. PHAGOCYTOSIS OF NEUTROPHILES BY  
MACROPHAGES IN PERITONEAL EXUDATE.  
(PHASE CONTRAST ILLUMINATION)

When an inflammatory exudate is induced in a guinea pig by intraperitoneal injection of sodium caseinate solution, the predominant type of phagocyte during the initial phase is the neutrophile. By the 24th hour, many of the neutrophiles have been ingested by the increasing number of macrophages which are mobilized; this is the apparent fate of all senile or damaged neutrophiles. The ingested neutrophile becomes surrounded by a refractile vacuole; the multilobulated nucleus becomes pyknotic, and the cytoplasmic constituents become progressively condensed into a homogeneous appearing mass (Figure 13).

If virulent phagocytosis-susceptible P. pestis cells are injected into guinea pigs 24 hours after inducing a sterile peritoneal exudate, many neutrophiles containing the bacillus may be observed within macrophages as seen in Figure 14. When a neutrophile containing P. pestis is ingested by a macrophage, the neutrophile shows all the signs of necrosis and digestion while the bacteria continue to appear normal.

Figure 15 under phase microscopy shows two macrophages containing neutrophiles. One neutrophile is in an early stage of digestion, and one is in a late stage.

Figure 16 is the same preparation at a slightly different focal plane showing normal appearing P. pestis in the vacuole occupied by the neutrophile remnant.

Many macrophages in exudates infected with P. pestis 24 hours or more before sampling contained remnants of neutrophile cytoplasm and nuclei in large vacuoles that also contained P. pestis. Many P. pestis cells were also observed within macrophages which were not encased in vacuoles. When P. pestis cells were contained in large vacuoles there was almost always some evidence of neutrophile debris in the same vacuole (Figure 17).

Many of the macrophages in old plague infected exudates were filled with what appeared to be actively growing P. pestis with no evidence of any large digestive vacuoles (Figure 18).

By this time any extracellular organisms would be resistant to further phagocytosis, so it may be assumed that the intracellular organisms were progeny of the original susceptible type ingested earlier. If the susceptible type were taken up directly by a macrophage they apparently multiplied quite rapidly and were not encased within vacuoles, or, if they were, the vacuoles was not much larger than the individual bacterium. However, if the susceptible type were originally taken up by neutrophiles, and these phagocytes then were ultimately taken up by macrophages, it is our impression that the bacteria were released by digestion of the neutrophile and then were able to grow within the large vacuole containing the remnants of the neutrophile, since large numbers of normal appearing P. pestis were seen within the large cytoplasmic vacuoles of macrophages which result from ingestion of neutrophiles.



FIGURE 14. MACROPHAGE WHICH HAS INGESTED A NEUTROPHILE CONTAINING P. PESTIS ORGANISMS. (GIESMA)



FIGURE 15. MACROPHAGES CONTAINING NEUTROPHILES IN DIFFERENT STAGES OF DIGESTION. (PHASE CONTRAST ILLUMINATION)

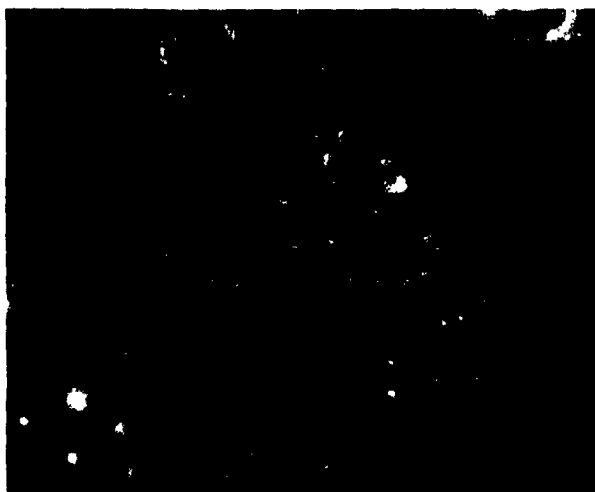


FIGURE 16. SAME MACROPHAGES AS FIGURE 15, AT DIFFERENT FOCAL PLANE, SHOWING P. PESTIS CONTAINED WITHIN PARTIALLY DIGESTED NEUTROPHILE.



FIGURE 17. MACROPHAGE CONTAINING P. PESTIS AND NEUTROPHILIC DEBRIS. (Giemsa)



FIGURE 18. MACROPHAGE CONTAINING ACTIVELY  
GROWING P. PESTIS. (GIEMSA)

In summary, circumstantial evidence indicates that the hypothesis which states that "the ability of P. pestis to resist phagocytosis is the major determinant of its virulence" must be re-evaluated. We suggest that the major determinant of the virulence of P. pestis is its ability to survive and multiply within the phagocytic cells of the host defense system.

## REVIEW OF PLAGUE

### THE PLAGUE PROGRAM 1962-63, USAMU, FOREST GLEN

Raymond Randall, DVM\*

Experimental evidence indicates that vaccination against pneumonic plague with presently available killed or living attenuated vaccines provide, in guinea pigs and monkeys at least, a very uncertain method of protection against aerosol challenge; therefore, search for an adequate and safe plague vaccine suitable for man will be continued.

In this approach different species of animals will be immunized with different combinations of the antigens of Pasteurella pestis or with attenuated live vaccines using either peripheral or aerogenic routes for vaccination. Before respiratory or peripheral challenge the animals will be bled to determine the significance of different types of humoral antibody as stimulators of acquired resistance. This will require the production, isolation and purification of the antigenic components of P. pestis, especially those other than Fraction I and toxin in order to show which are determinants of virulence and act on the humoral and sessile phagocytic systems.

Further, it is strongly indicated that certain living plague vaccines elaborate a diffusible protective substance in the host which is not produced with the present accepted methods for cultivation of P. pestis in vitro; therefore, continued attempts are to be made to isolate and characterize this protective factor from in vivo- and in vitro-grown P. pestis.

a. In a continuation study of the pathogenesis and therapy of plague in animals, experiments will be conducted on the response of nonimmunized and immunized animals to the different strains of P. pestis that lack one or more of the several recognized antigens.

b. The screening of new antibiotics and other therapeutic agents against plague will include dosage and the time factor in the employment of the agents.

\* Walter Reed Army Institute of Research, Washington, D. C.

## REVIEW OF PLAGUE

### SEROLOGICAL MICROTECHNIQUES FOR THE DETECTION OF SPECIFIC PASTEURELLA PESTIS ANTIBODY: THE APPLICATION OF THESE TECHNIQUES TO ECOLOGICAL AND EPIDEMIOLOGICAL PLAGUE STUDIES.

Raymond Randall, DVM\*

The well recognized serological procedures for the identification of specific Pasteurella pestis antibody have been described in the literature. Two are the complement fixation (CF) and hemagglutination (HA) tests, developed by the Meyer group, which employ the purified capsular antigen of the plague bacillus, Fraction I (Baker, E. E., Sommer, H., Foster, L. E., Meyer, E., and Meyer, K. F., Proc Soc Exper Biol & Med 64:139-141, 1947; J Immunol 68:131-145, 1952; Chen, T. H. Quan, S. F., and Meyer, K. F., J Immunol 68:147-158, 1952; and Chen, T. H., J Immunol 69:587-596, 1952). These tests as described are simple and reliable, but are not especially suited for the field and have generally necessitated the collection and shipment of serum specimens to a distant laboratory. Transportation of sera is not always satisfactory, due to the great distances some specimens must be shipped and, in certain areas, the difficulties encountered in the collection of proper specimens. In view of the desirability of performing the serological examinations locally, we have undertaken a study of some newly developed microtechniques and the possible application of these techniques to plague research.

Some of the procedures developed by Casals of The Rockefeller Institute and Weinbren of the East African Virus Research Institute, Entebbe, are satisfactorily adapted to the CF and HA tests with Fraction I antigen, but these tests still require considerable elaborate equipment for efficient use and are not adaptable for general field work.

The microserological procedures employing small plastic plates instead of test tubes, calibrated dropping pipettes, and transfer loops for serial dilutions were first introduced by Dr. Takatsy, of Hungary, in 1950. A subsequent paper by Sever (Sever, J. L., J. Immunol 88:320-329, 1962) has applied this general equipment to various other tests and has incorporated modifications which improve the efficiency of the original system. We have adapted the standard CF and HA tests to this microsystem. The equipment has also been applied to other serological procedures, as yet under study, employing purified Fraction I adsorbed on Bentonite particles (Bozicevich, J., Proc Soc Exper Biol & Med 97:180-183, 1958) or formalinized red blood cells (McKenna, J. M., Proc Soc Exper Biol Med 95:591-593, 1957; Csizmas, L., Proc Soc Exper Biol Med 103:157-160, 1960).

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## MATERIALS AND METHODS

**Antigen.** The antigen employed in these studies was purified Fraction I prepared according to the method of Baker, et al (1947, 1952). The multiplicity of *P. pestis* antigens (Lawton, W. D., Fukui, G. M., and Surgalla, M., J. Immunol 84:475-479, 1960) indicate the desirability for strict control of purity prior to their use in extremely sensitive tests. A slide was shown of an Ouchterlony plate (Thorne, C. B., and Belton, F. C., J Gen Microbiol 17:505-516, 1957) in which the thrice precipitated Fraction I in 1 mg/ml concentration had been tested against Lederle's polyvalent antiplague gamma globulin. Ten ml of the Fraction I solution, containing 5 mg/ml were dried in ampules and stored. Dried Fraction I was rehydrated with 10 ml of phosphate buffered saline pH 7.4 prior to use in the tests.

**Equipment.** The Microtiter equipment in use was purchased from the Cooke Engineering Co., Alexandria, Va.

### Procedures.

1. **The Hemagglutination Tests.** The procedure of Chen (1952) was followed in sensitizing tanned red blood cells with Fraction I and were extended to include the sensitization of red blood cells with formalin.

In the HA tests, nonspecific inhibitors are adsorbed as follows: One-tenth ml of a 10% suspension of sheep red blood cells to be used in the tests was placed in a 6 x 50 mm test tube. One transfer loop, of the inactivated test serum (0.025 ml, was then mixed with the suspension of red blood cells, resulting in a 1.5 dilution of the serum. The serum-blood cell suspension was then incubated at 37 C for 30 minutes. The red blood cells were sedimented by placing the small test tubes in a Babcock centrifuge cup and centrifuging them at 1,500 rpm for 10 minutes. The supernatant serum dilution was then removed with a transfer loop and provided the first serial dilution.

Using the calibrated dropping pipette, 0.025 ml of diluent (1% normal rabbit serum in saline) was added to each well of the test plate. Serial, 2-fold serum dilutions were then made, using the 0.025 ml transfer loop. One drop of antigen, consisting of a 0.01% suspension of tanned or formalinized red blood cells, sensitized with Fraction I in a 1% solution of normal rabbit serum in saline, was then added to each well. The plates were then sealed with Scotch tape gently agitated and incubated overnight at room temperature. The tests were then read with the aid of a magnifying mirror. Positive tests consisted of a thin layer of agglutinated red blood cells covering the entire bottom of the well, as may be observed in the macroscopic tube test.

2. **The Complement Fixation Test.** Again, the procedure followed was that of Chen (1952) by incorporating only those deviations required for a micro-test. Complement, antigen and amboceptor were titrated as for the

standard test for syphilis and as the amounts of reagents utilized in the tube test were proportional, the test was performed using the dropping pipettes and transfer loops. One drop, 0.025 ml, of Kolmer saline was placed in each of the wells of the test plate. Inactivated test serum was taken up with a transfer loop and serial, 2-fold dilutions of the serum were made. Simultaneous dilutions of serum for the AC control were made at this time. One drop (0.025 ml) of complement determined by titration to contain 2.5 full units was then added to each well and the plate gently agitated. One drop (0.025 ml) of Fraction I antigen was added to each well of the test dilutions while a drop of Kolmer saline was added to each of the AC control dilutions. The plate was then covered with Scotch tape, gently agitated and placed at 5 C overnight. The following morning, two drops (0.05 ml) of the hemolytic system were added to each well of the test after the plates had been prewarmed to 37 C for one hour. The plates were again sealed with tape and placed, after vigorous agitation, at 37 C. It was found necessary to agitate the plates vigorously every 5 minutes to prevent the appearance of false AC reactions.

3. Bentonite Flocculation Tests. The above described Fraction I antigen was substituted for DNA used in the original procedure for adsorbing antigen onto bentonite particles, prepared as described by Bozicevich (1958). Serial 4-fold dilutions of inactivated serums were prepared in a stabilizing diluent that was found to be necessary for the test. The diluent consisted of 0.8 gm bovine serum albumin (Armour) in 100 ml of normal saline. A further, most important requirement for this test, is that the serum specimen must be fibrin free, to prevent false-positive reactions. Each serum dilution was removed with a Pasteur pipette and placed on a plastic plate of the Huddleson type, subdivided into 1 inch squares. One drop of the Bentonite antigen was added to each serum dilution and mixed with a toothpick. The plate was gently rocked on a variable speed rotator and then observed over transmitted light for the appearance of flocculation. The rule established by experiment trial was that the plate should be rocked for 5 minutes and then read. Following the test, the plate was washed with water and was ready for re-use. This Bentonite antigen when held at room temperature for 3 months has remained stable for this period of time.

## RESULTS

### Application of Microserological Procedures to Sera Obtained in a Survey of Plague Endemic Regions in New Mexico.

Plague was first observed in the state of New Mexico in 1938, when prairie dogs and field mice were found dead in Catron County. Since 1938, plague has been found to be enzootic in much of the state. As observed in India, periodic epizootics have resulted in the appearance of plague-resistant rodents among formerly susceptible species in the enzootic areas, although, the rodents in apparently nonenzootic areas still die in massive numbers when plague is introduced. The mountainous regions surrounding the city of Santa Fe appear to harbour resistant species while the arid Sonoran plateau region typified by Roswell, has recently been afflicted with an extensive epizootic.

Table I lists the location, date, life zone and elevation of the 12 known cases of human plague which have occurred in New Mexico since 1949 when plague was first reported in man. Unfortunately, the source of infection cannot be given in many of the cases inasmuch as the exact whereabouts of the contacts are unknown. Plague in animals, however, has been proven to exist in close proximity to the areas in which the human subjects worked or lived.

TABLE I. KNOWN HUMAN PLAGUE IN NEW MEXICO.

NO. HUMAN INFECTIONS	DATE	TOWN AND LOCATION IN NEW MEXICO		ELEVATION (feet)	LIFE ZONE
1	1/7/59	Sandia Park	N	7,500	Transition
1	26/7/49	Cerro	N	7,000	Transition
1	28/7/49	Placitas	N	6,000	Upper Sonoran
1	4/7/50	Glorietta	N	7,000	Transition
2	21/7/61 8/8/61	Pecos	N	9,000	Canadian
1	29/7/61	Santa Fe	N	7,000	Transition
1	5/11/49	San Patricio	S	5,500	Upper Sonoran
1 <sup>a/</sup>	6/1/50	Maljamar	S	3,200	Lower Sonoran
1 <sup>a/</sup>	2/1/51	Hobbs	S	3,625	Upper Sonoran
2 <sup>a/</sup>	19/2/60 20/2/60	Roswell	S	3,500	Upper Sonoran

a. Rabbits incriminated in the case.

It can be observed in this table that plague occurs or can occur throughout the year in New Mexico. In the winter months, the Southeast portion of the state may be expected to produce cases, the potential in this area appears to decrease with the advent of hot summer weather. Further, the disease in this area appears to be closely associated with rabbit plague although the initiating focus appears to be in the prairie dog, pack rat, or ground squirrel, etc., through their close association with rabbits in the Sonoran ecological situation. Plague infection appears to be caused by contact infection here, as with tularemia.

By contrast, the North Central plateau with its high mountains produces ideal climatic requirements for the support of epizootics during the spring and summer months. Plague again disappears during the hot summer months in the lower (5,000-7,000 ft) portions while it usually just appears in the higher (9,000-12,000 ft) regions. With the advent of fall and winter, the infection then subsides in the higher mountains and may reappear in the Southeastern portion of the state.

The first human case in the 1959-61 series was a small girl, infected about 1 July 1959, in the high mountainous area just outside Albuquerque. No animal was incriminated with certainty in this case, although an association with rabbits is suspected, since the child played with a "trapped" wild rabbit and several rabbits were shot and then dressed at the family home. The family dog was ill at the same time as the child and was shown to have developed a high HA antibody titer shortly after the child died. This child was an Air Force dependent, and was seen at the out-patient clinic of Sandia Air Base Hospital, where plague was diagnosed postmortem.

The second case in this series occurred in February 1960 when two Air Force officers attached to Walker Air Base went rabbit hunting in the vicinity of Roswell. They had excellent hunting, cleaned several rabbits, returned to the Air Base and showed the first symptoms of plague infection the day prior to their contemplated departure to London on a SAC mission. Plague was included in the tentative diagnosis of these cases inasmuch as the symptoms of the disease followed that of tularemia described in the consulted references. These patients, who made uneventful recoveries after adequate treatment, were instrumental in initiating the epidemiological survey which followed. In this case, the survey workers were led directly to the site where rabbits had been dressed in the field. The rabbit skins were recovered and found to be positive for P. pestis. Numerous rabbits, pack rats, etc. were found dead in the immediate area and several were also positive. The epizootic appeared to be very extensive, dead rabbits being reported for many surrounding miles; one jack rabbit being found on a Walker Air Base landing strip some 35-40 miles from the site where the two officers had become infected.

In 1961, three cases of plague were reported in the Santa Fe area of New Mexico. A lumber worker died with a tentative diagnosis of fever type undetermined in July, 1961, followed, in succession by a geologist who was infected in the Santa Fe region, and died in Boston where he had traveled by air. Further, a case in a linesman who recovered after adequate treatment due to the knowledge gained from the death of the two preceding cases. An unfortunate delay in the identification of P. pestis isolated from the first case resulted in a very cold trail for epidemiologists to follow. Further, fall had set in and many of the possibly infected carrier animals in the mountains had gone into hibernation. Fortunately, P. pestis was isolated by Mr. F. M. Prince of the Public Health Service in the work area of the linesman's infection from the tissues of two ground squirrels, early in September at an altitude of about 10,000 feet.

In late September and early October, a team from the Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, consisting of Captain D. C. Cavanaugh, Dr. J. H. Rust and Sp5 W. Sunderman, conducted a survey in the Roswell and Santa Fe areas. Live traps were used to obtain animals from which sera, tissues and ectoparasites were obtained for study. Intensive trapping in the area of the linesman's case was unrewarding due to the advent of fall, with snow, and the previous trapping done by Mr. Prince. In all, some 284 small rodents were trapped and bled in other suspect Santa Fe areas. A trip to the Roswell area which had undergone an extensive epizootic 18 months previously yielded 46 specimens of sera from assorted rodents and rabbits; the animals were bled either from the orbital sinus or the heart.

The geologist had been accompanied by other professional workers, and his death did not deprive us of information as to the ground he had covered. These individuals were later located at their various home stations throughout the United States and specimens of blood were obtained, with histories as to exposure, recent illness, and previous vaccination against plague, etc. All of these 5 men had been in close association with the geologist, but none had been clinically ill during this time period and none had received previous plague immunizations.

P. pestis was not isolated from any of the specimens of animal tissues or ectoparasites by culture or by animal inoculation. Nor were any tissues positive by the fluorescent antibody technique when tested by Mr. Prince in the San Francisco Laboratory.

Table II lists the serological results obtained when sera were tested by the CF and HA microtechniques. Prior to testing, all sera were inactivated at 62 C for 20 minutes. From 249 sera obtained in the Santa Fe area, one positive result was obtained in the HA test. This serum was taken from Peromyscus truei trapped in the work area of the geologist, seven miles outside of Santa Fe in Arroyo Frijoles. This serum gave a positive reaction in four repeat tests, in a titer of 1:80. All sera were negative in the CF tests. The 45 sera shown in Table III were obtained in the Roswell area and produced 3 positive HA reactions. These sera were taken from Sylvalagus (titer 1:1280), Onychomys leucogaster (titer 1:40) and Dipodomys ordi (titer 1:20). From Table II, it may be seen that many rodent species were tested by these methods. The large number of negative results with the HA tests, and the negative, and not anticomplementary, CF tests indicate that these tests will be of great use as supplements to classical survey procedures in areas where it appears that plague infection in our western states is maintained in small, discrete foci, comprising the restricted range of small mice or other rodents. These foci may initiate epizootics under proper conditions and thus serve as population controls in nature. In such areas, numerous rodents must be tested to obtain positive serological tests, unless the exact limitations of the foci are known. In other areas, which have undergone extensive epizootics, better results may be expected. In such areas, Roswell for example, the rodent population had been decimated by plague

TABLE II. RESULTS OF SEROLOGICAL TESTS FOR PLAGUE ANTIBODIES IN SERA FROM ANIMALS TRAPPED NEAR SANTA FE, NEW MEXICO.

SPECIES	NUMBER TESTED	NUMBER POSITIVE
<u>Neotoma albigula</u>	62	0
<u>Peromyscus truei</u>	46	1
<u>Dipodomys ordi</u>	40	0
<u>Peromyscus sp.</u>	30	0
<u>Eutamias minimus</u>	19	0
<u>Sylvilagus sp.</u>	6	0
<u>Clethrionomys</u>	6	0
<u>Sciurus sp.</u>	5	0
<u>Citellus sp.</u>	4	0
<u>Perognathus sp.</u>	2	0
<u>Canis sp.</u>	2	1

TABLE III. RESULTS OF SEROLOGICAL TESTS FOR PLAGUE ANTIBODIES IN SERA FROM ANIMALS TRAPPED NEAR ROSWELL, NEW MEXICO.

SPECIES	NUMBER TESTED	NUMBER POSITIVE
<u>Dipodomys ordi</u>	22	1
<u>Onychomys leucogaster</u>	10	1
<u>Neotoma albigula</u>	5	1
<u>Sigmodon hispidus</u>	3	0
<u>Sylvilagus sp.</u>	2	1
<u>Lepus sp.</u>	2	0
<u>Dipodomys bancrofti</u>	1	0

and at the time of our survey, rodents had not re-established themselves in numbers. Yet, apparently, infective fleas had remained in sufficient numbers to produce some sub-clinical infections in a goodly number of the indigenous animals.

Of the human sera collected in conjunction with the case of the dead geologist, one positive specimen was obtained. This specimen came as a whole blood specimen and was anticomplementary, thus the CF test was not satisfactory. The individual, however, was positive at 1:1280 in the HA test and 1:258 in the Bentonite flocculation test. It may be assumed from this that he had been infected at the same time as the geologist, but had aborted the infection, as has been shown by Payne and McCrumb, and others, to occur in individuals in Madagascar. Unfortunately, further serological studies are impossible with this individual, due to his immunization against plague shortly after the blood specimen was obtained.

#### Results with Bentonite Flocculation.

It was not possible to test many of the above mentioned sera with the Bentonite method as they had been used up by the time the Bentonite test was developed. A large store of sera, however, which had been obtained in Madagascar by Drs. McCrumb and Payne from their pneumonic plague patients were available for testing. Several consecutive serum specimens were in this series and were tested with the results shown in Table IV, in which the HA and Bentonite flocculation tests are compared. These sera are all anticomplementary and the CF test could not be performed. It may be seen that good titers were obtained with serum from individuals who have been infected with plague. These cases show a rather rapid rise and decline reminiscent of the bacterial agglutination test. The Bentonite reaction does not appear to persist as does the hemagglutination antibody, nor do the two tests correlate in titer. Further, vaccinated individuals as shown in Table V do not respond too well with flocculating Fraction I antigen sensitized Bentonite particles. The positive Bentonite tests with sera obtained from contacts as shown in Table VI is interesting, inasmuch as both the HA and Bentonite tests show some exposure to P. pestis must have occurred in these contacts, both in the Madagascar series as well as in one of the geologist's group from New Mexico.

The Bentonite antigen has been prepared in several lots and appears to be extremely stable, requiring no refrigeration or special treatment. A further advantage is that no adsorption of sera is required and no heat labile reagents or elaborate equipment are required. We are continuing our studies with this type of antigen in an effort to relate it to the other established procedures in order to ascertain the niche it may fill in plague research.

Our studies with the microequipment described above lead us to conclude that, in the field of plague research, tests performed with carefully prepared reagents are reproducible and reliable. In areas where equipment is

TABLE IV. BENTONITE FLOCCULATION TESTS IN PNEUMONIC PLAGUE INFECTION.

CASE NUMBER	DAY OF DISEASE	BENTONITE FLOCCULATION TITER	HEMAGGLUTINATION TITER
1	21	256	20480
	36	1032	81920
	42	1032	81920
2	15	1032	2560
	30	1032	10240
	45	1032	5120
	74	256	5120
3	17	1032	162840
	32	256	162840
	47	256	162840
	76	4	81920
4	15	64	20
	39	4	40

TABLE V. RESULTS OF BENTONITE FLOCCULATION IN INDIVIDUALS IMMUNIZED WITH EV76.

CASE NUMBER	DAY OF TEST	BENTONITE FLOCCULATION TITER	HEMAGGLUTINATION TITER
1	0	-	--
	14	16	320
	42	256	160
2	0	-	40
	14	256	640
	42	±	640

TABLE VI. RESULTS OF BENTONITE FLOCCULATION IN INDIVIDUALS WHO WERE CONTACTS OF PNEUMONIC PLAGUE CASES.

CASE NUMBER	DAY FOLLOWING CONTACT <sup>a</sup>	BENTONITE FLOCCULATION TITER	HEMAGGLUTINATION TITER
1	2	256	10
	9	256	640
2 <sup>b</sup> /	5	±	0
	15	64	5120
3	2	16	4032
4	9	16	101280
5	9	64	Less than 10

a. Day of onset of disease in contact.

b. Became febrile, treated for plague.

short, as in the field or in meagerly equipped laboratories in underdeveloped areas, suitable diagnostic serological procedures required for a more thorough work-up of plague epidemiology may now be performed. In areas where the quantity of reagents, particularly sera from small rodent species, is critical, the fact that 4 tests can be performed with 0.1 ml of sera is very significant and permits the investigator to perform at least three separate tests, the CF, HA, and Bentonite flocculation, on each specimen with enough sera remaining to repeat equivocal tests. The fact that stable, dry capsular antigen may be prepared in suitable laboratories and shipped to the field as a standardized reagent for CF and HA tests is a useful bit of knowledge. The potentially useful Bentonite test is also under consideration as to its stability under adverse storage conditions.

## REVIEW OF PLAGUE

### IMMUNIZATION OF MAN AGAINST PLAGUE

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Systematic observations at the Hooper Foundation over several years on clinical and serologic reactions of human volunteers to various plague antigens and vaccines have been reviewed.

The inoculations of the antigen in two doses 14 to 30 days apart have caused very few severe side-effects. Concentrations exceeding 3 billion organisms per milliliter increase the number and severity of the local and occasionally systemic reactions. In the customary dose of 1 billion partly living and partly dead avirulent plague bacilli - of the EV 76 strain, the systemic reactions after subcutaneous inoculations between the shoulders have been general malaise, temperatures of 98.6 to 103.6 F and incapacitation for up to 72 hours. Subcutaneous inoculations of envelope Fraction I antigen in doses estimated on a weight basis to represent the Fraction I content of 20 billion dead plague bacilli have been well tolerated by groups of civilian but not military volunteers. The second and third inoculations have caused some marked local erythematous reactions with edema extending to the forearm.

Inoculation of aluminum-hydroxide adsorbed or precipitated formalin-killed virulent plague bacilli in the dose of 0.3 ml containing 1 billion cells caused no severe side-effects. Concentrations exceeding 3 billion have increased the number and severity of the local reactions.

Basic immunizations with antigen inoculations 14 to 30 days apart with few exceptions have stimulated the appearance of antibodies in the peripheral blood in modest titers. The mouse protection indices (MPI) 1 month after the second inoculation have not been spectacular. The envelope Fraction I antigen and the aluminum-hydroxide adsorbed formalin-killed whole virulent Pasteurella pestis preparation have induced average MPI titers below 10, hemagglutination titers above 100 and in a few individuals complement-fixation titers.

As pointed out in 1953, all of our work supports the idea that booster reinoculation is an essential part of the immunization against plague. The serologic response to the third inoculation with relatively small doses of antigen or vaccine has been vigorous as a rule. Whole plague bacilli or the immunogenic envelope antigen sensitizes the tissues to such an extent that the booster effect influences all the antibodies. Most of the MPI have been 10 or lower after the booster. When the response to basic immunization with relatively inactive antigens has been slight, the booster effect of the third inoculation has also been slight; for example, the secondary or tertiary

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response in volunteers immunized with aqueous suspensions of formalin-killed virulent plague bacilli - the Army vaccine - has been slightly inferior to that induced by alum-adsorbed antigens.

Within 3 months, and definitely by the 6th month after either basic or booster inoculations, the antibody levels have declined. Presumably the potential immunity persists, and the response to second and third booster inoculations is definite. In fact, the immunity measured by protective antibodies has been maintained at a fairly constant level by re-inoculation at 3 month intervals. Furthermore, observations on human volunteers who had received Army vaccine during the past 8 to 16 years indicate that sensitization to plague antigen persists and the antibody response to re-inoculation is vigorous and occasionally spectacular.

Controlled data on the effectiveness of plague vaccines are virtually non-existent. The serologic studies on volunteers strongly support the conviction that vaccines preferably with synergists will serve as a major means of reducing the risk of infection.

## REVIEW OF TULAREMIA

### INFECTIVITY OF AGED AEROSOLS OF PASTEURELLA TULARENSIS

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Recently increasing attention has been devoted to assessing the influence of environmental factors upon various properties of aerosolized microorganisms. For example, factors such as ultraviolet and visible light, temperature and relative humidity have been studied as to their influence upon microbial aerosols. A number of observations have been conventionally included in any sophisticated characterization of the properties and behavior of such aerosols. Some of these are (1) efficiency of the aerosol fixture; (2) original concentration of viable airborne organisms; (3) characterization of the aerosol particles with respect to size and microbial population; (4) physical decay due to phenomena such as dilution, gravity, agglomeration and the like; (5) biological decay, that is the rate of loss of viability, and (6) where infectious organisms are concerned, the inhaled dose-response curve. Conspicuously absent have been characterizations of infectivity decay.

At the risk of over-simplification I should like to define the terms being employed. Viable indicates the ability to replicate in or on a suitable medium and infectious indicates the ability to invade and multiply detectably in appropriate hosts; in general, in the laboratory these are reduced to counting colonies on plates as compared to sick animals.

That viability and infectivity of pathogenic microorganisms might not always be equated has been suspected for sometime. Perry demonstrated during the course of epidemiological studies of acute streptococcal infection, reported by Rammelkamp in 1956, that individuals deliberately exposed to viable streptococci in heavily contaminated environments, as well as to artificially contaminated dust, did not develop disease. It was suggested that environmental factors such as drying altered the organisms so that they failed to grow in the respiratory tract. More recently, Schlamm's data comparing assay methods for aerosolized Pasteurella tularensis provided quantitative evidence of dissociation between viability and infectivity. He found that aerosols of 6 or more hours age retained less than 1/10 the infectivity of newly created aerosols for mice directly inhaling the aerosol. Collection of the aerosolized organisms and intraperitoneal (IP) inoculation into mice, however, failed to indicate a loss in infectivity; that is the LD<sub>50</sub> so measured was the same for fresh and aged aerosol organisms. At about the same time, and working independently, Hood confirmed Schlamm's findings in mice though he felt that a somewhat longer period of time was required. When Hood duplicated the studies in guinea pigs, he again encountered a dissociation between viability and infectivity of P. tularensis with prolonged aerosol age. However, in this case it was reflected in both

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directly exposed animals and in those inoculated IP with organisms collected from the air. Hood also noted a delay in time to death and an apparent decrease in severity of illness in animals infected by cells from the aged clouds.

Goodlow and Leonard have reported that aging of aerosols of both P. tularensis and Pasteurella pestis resulted in loss of infectivity for directly exposed guinea pigs out of proportion to the loss of viability. The elapsed times employed were 5½ hours in the case of tularemia and 40 minutes for plague.

These observations with P. tularensis in laboratory rodents established that infectivity and viability may dissociate as a function of the duration of the airborne state. That this could be true for man as well was suggested by the observations reported to this group last year. Those data were derived during comparisons of the infectious dose of aerosolized living attenuated tularemia organisms with that of the virulent SCHU-S4 strain. Volunteers were exposed in such a way that they inhaled approximately 200 viable cells of the SCHU S4 strain which had been aerosolized 30 minutes earlier. To our surprise but 4 of the 8 became ill.

The reason for our surprise is seen in Table I. Previous experience in volunteers with aerosolized SCHU-S4 was accomplished by Saslaw and by McCrumb and his associates utilizing equipment in which the aerosol was but a second or so old when inhaled. The ID<sub>50</sub> for man was 10 or less cells. On this basis we expected all the volunteers inhaling 200 cells to be ill. They weren't. Moreover, those that did become ill did so after incubation periods of from 4 to 8 days whereas the incubation periods in those infected by fresh aerosols fell in the 3- to 5-day range.

TABLE I. RESULTS OF HUMAN EXPOSURE TO AEROSOLIZED P. TULARENSIS SCHU-S4 STRAIN.

NUMBER OF ORGANISMS INHALED		NUMBER INFECTED/NUMBER EXPOSED	
		"FRESH" AEROSOL	"AGED" AEROSOL (30 minutes)
10 -	20	7/10	
20 -	40	4/4	
40 -	55	5/6	
170 -	260		4/8
225 -	335	4/4	
1,300 -	30,000		

This, then, could represent in man the phenomenon described earlier as occurring over several hours time in the case of laboratory rodents. Variables among the generators and other equipment inherent in the comparison could account for all the differences observed. This, however, seemed less likely and subsequent results have indicated the hypothesis to be tenable, namely that with increasing age as an aerosol P. tularensis loses infectivity for man while retaining viability.

Five separate cultures of the SCHU-S4 strain of P. tularensis have been used. All were prepared by Dr H. T. Eigelsbach and were similar in concentration, physical characteristics and guinea pig virulence. With but a single exception all trials were conducted between the 14th and 21st days following harvest. Aerosols were generated within a spherical static chamber of 1,000,000 liter volume employing a single 2-fluid nozzle generator. Temperature and relative humidity were optimal for aerosolization of this organism. Source strengths and decay rates were similar in all tests. Aerosol concentration was determined by impingement in AGI30 samplers with British pre-impingers and plating on suitable media. Of the total viable cells, in excess of 60% were contained in particles 5  $\mu$  or less in diameter.

Concurrently with the studies in man, simians were exposed to the different aged aerosols. The infectivity at 60, 120 and 180 minutes cloud age is shown in Table II. The inhaled doses of viable organisms are grouped arbitrarily with consideration for the techniques employed. By inspection, any alternate form of grouping would not alter the interpretation. There is a dissociation of infectivity and viability with increase in aerosol age. At low doses, the change is detected at 120 minutes and is marked at 180 minutes. It should be emphasized, however, that increase of inhaled dose by 10-fold or less resulted in an incidence of infection similar to that from younger aerosols.

Monkeys were considered infected on the basis of unequivocal evidence in two or more of the following categories. prolonged fever and discernible clinical illness, positive cultures, radiographic evidence of pneumonitis, a 4-fold or greater rise in agglutination titer, and compatible autopsy findings. No therapy was administered.

In general, infected animals did not differ markedly from group to group though there was a tendency toward prolongation of the incubation period in animals exposed to older aerosols. No subclinical infection was detected.

Mortality within 30 days of exposure (Table III) in general confirmed the clinical impression about the similarity of disease in the various groups. With the 180-minute-old aerosols there was a suggestion that mortality was lessened even at relatively high dosages. However, animals apparently surviving infection have been sacrificed up to 60 days later and found to have extensive gross and histologic lesions of tularemia. Within the limits of this study, it appeared that the disease, once induced, was similar and ran a comparable course regardless of the age of the aerosol inducing it.

TABLE II. INFECTIVITY OF AGED AEROSOLS OF P. TULARENSIS (SCHU-S4)  
FOR MACACA MULATTA

VIABLE CELLS INHALED		NUMBER INFECTED/NUMBER EXPOSED		
		Aerosol Age (Minutes)		
		80	120	180
9 -	100	7/8	1/8	1/19
100 -	200	7/8	10/12	2/20
200 -	500	12/12	11/11	1/16
500 -	1,000	12/12	7/8	4/6
1,000	13,000	16/16		15/17

TABLE III. MORTALITY FROM INFECTION OF M. MULATTA BY AGED AEROSOLS  
OF P. TULARENSIS (SCHU-S4).

VIABLE CELLS INHALED		NUMBER INFECTED/NUMBER EXPOSED		
		Aerosol Age (Minutes)		
		60	120	180
9 -	100	5/7	1/1	1/1
100 -	200	6/7	6/10	0/2
200 -	500	8/12	7/11	1/1
500 -	1,000	7/12	6/7	2/4
1,000 -	13,000	16/16		8/15

Guinea pigs were inoculated subcutaneously with aerosolized organisms collected in impingers. The procedure was complicated by the low aerosol concentrations employed. Nevertheless, the data for 4 trials (Table IV) are consistent with those of previous workers in that slight but definite increases in LD<sub>50</sub> were noted in the older aerosols. The time span was shorter, however, than those employed in other studies.

The infectivity of the aged aerosols as determined in volunteers is shown in Table V. Illness was determined by clinical criteria and in no

TABLE IV. INFECTIVITY OF AGED AEROSOLS OF P. TULARENSIS (SCHU-S4)  
GUINEA PIG SUBCUTANEOUS TITRATION OF SAMPLERS.

TRIAL	LD <sub>50</sub> ( <u>P. tularensis</u> )		
	Aerosol Age (Minutes)		
	60	120	180
I	1-4		>40
II	1-3	3-5	5-10
IIIA	1-3	3-5	
IIIB	3-5	5-10	
IV			16

TABLE V. INFECTIVITY OF AGED AEROSOLS OF P. TULARENSIS (SCHU-S4)  
FOR MAN.

VIABLE CELLS INHALED	NUMBER INFECTED/NUMBER EXPOSED			
	Aerosol Age (Minutes)			
	30	60	120	180
80 - 150			0/4	0/4
170 - 350	4/8	3/4	1/4	0/8
700 - 1,000		4/4		0/4

case was there discordance with serological response. It is evident that infectivity for man digressed from viability as a function of aerosol age just as it did in simians and rodents.

The disease induced did not differ markedly among the groups. Incubation periods were from 4 to 13 days. Those individuals with incubation periods in excess of 5 days characteristically experienced a 1- to 2-day period of vague symptoms prior to the onset of overt typhoidal-type tularemia. All but one became ill and were treated with streptomycin. Response was prompt. One individual, exposed to the middle dose at 60-minutes cloud age, was but minimally ill for 24 hours and recovered without therapy. He had the longest incubation period of the group.

As men and rhesus monkeys were at times exposed to the same aerosols, a direct comparison of infectivity was possible (Table VI). Whereas previous tables indicated the number of viable organisms inhaled, the parameter dealt with here is the concentration of viable organisms in aerosols and not inhaled doses per se. Within each group of aerosol concentrations, results are presented for men exposed for 1 minute and rhesus monkeys, for 3 minutes and for 10 minutes. For reference purposes the respiratory minute volume of the rhesus is approximately 1/10th that of man; hence a monkey exposed 10 minutes inhales approximately the same number of organisms as a man exposed 1 minute and monkeys exposed 3 minutes inhale just under 1/3 the human inhaled dose.

TABLE VI. INFECTIVITY OF AGED AEROSOLS OF P. TULARENSIS (SCHU-S4) FOR MAN AND M. MULATTA.

GROUP	AEROSOL CONCENTRATION (Organisms/L)	EXPOSURE TIME (Minutes)	SPECIES	NUMBER INFECTED/NUMBER EXPOSED			
				Aerosol Age (Minutes)			
				30	60	120	180
I	3 - 15	1	Man			0/4	0/4
		3	Monkey			0/4	0/8
		10	Monkey			1/4	1/7
II	17 - 19	1	Man	2/4	3/4		0/4
		3	Monkey				0/4
		10	Monkey		7/8		0/4
III	24 - 30	1	Man	2/4			0/4
		3	Monkey				0/4
		10	Monkey		4/4	7/7	0/4
IV	33 - 39	1	Man			1/4	
		3	Monkey		7/8	4/4	0/8
		10	Monkey		8/8	4/4	1/8
V	54 - 60	1	Man				
		3	Monkey			6/8	2/4
		10	Monkey		4/4	7/8	4/4
VI	70 - 100	1	Man		4/4		0/4
		3	Monkey				0/4
		10	Monkey		8/8		0/4

The dissociation of viability and infectivity is indicated as one reads horizontally in the groups, increasing aerosol age is associated with reduced infectivity. Note particularly the aerosol concentrations of Groups II, III, IV and VI.

Although all 3 exposure times are not available at each point, some tentative correlations are suggested. In Groups I, IV and VI at cloud ages of 120 and 180 minutes, when monkeys exposed to an aerosol 3 minutes were not infected, men exposed for 1 minute were not infected. Conversely, where 3-minute exposed monkeys developed disease, Group IV at 120 minutes-cloud age, some of the men became ill. Moreover, when all, or nearly all

of monkeys exposed for 10 minutes became ill, at least some of the volunteers were infected; note Groups II and VI at 60 minutes and Group IV at 120 minutes.

The discrepancy of observations of the lower right hand corner, that is Groups V and VI at 180 minutes-aerosol age is unexplained. These results were from separate studies; even in retrospect we can not account for the differences except by suggesting that only small numbers were employed. However, that results in men and monkeys were in agreement, even though at variance with prior experience in simians, supports the correlation suggested for man and monkey.

Briefly summarized, these data suggest that the rhesus monkey is sufficiently sensitive to small particle aerosols of P. tularensis as to be infected by 1/3 the inhaled dose leading to infection in man. Hence, for aerosol testing purposes the rhesus, despite the difference in breathing volumes, may serve as an excellent prognosticator of human response. It seems unlikely that in the near future field or test chamber work will arise in which the requirements or techniques will exceed the 3-fold factor noted. For the data presented indicating the dissociation of infectivity and viability with aging of aerosols it is apparent that an infectious system must be included for valid testing of aerosols in which significant time elapses between generation and sampling.

Studies are presently in progress in rhesus monkeys aimed at defining the effect of aerosol age upon infectivity of the streptomycin-resistant SCHU-S5 strain of P. tularensis. Insufficient time has elapsed since the exposure to do more than suggest that dissociation between viability and infectivity does occur as a function of aerosol age. However, within the 3-hour period employed infectivity appears to have been degraded to a lesser degree than with SCHU-S4.

One could speculate at some length as to the reason or reasons for the dissociation of microbial viability and infectivity induced during a relatively brief period as a small particle aerosol. Those physical factors, ionic, osmotic and thermal shock, which are operative in the loss of viability consequent to aerosolization are very likely directly related. However, in terms of the biochemical and biophysical determinants of viability and of infectivity of individual microorganisms which may be independently or differentially susceptible to the effects of aging in the aerosolized state there is currently little or no direct information. Technically the acquisition of such important data may be difficult because of the mixed population of cells without obvious morphological markers, i.e., a mixture of viable noninfectious, viable infectious, and dead cells within the airborne particulates. Perhaps approaches such as the use of synchronous cultures or combined statistical and biophysical evaluations could be fruitfully employed. In addition to completing the work with the SCHU-S5 strain, we are planning 2 studies to extend the present observations. One is the exposure of volunteers to approximately 10,000 viable cells of 180 minutes-aerosol age; as before monkeys will be exposed to the same

clouds for 3 and 10 minutes. Also, we plan to expose volunteers vaccinated with attenuated living organisms to large numbers of aerosol aged P. tularensis in order to compare the protection afforded with that against "fresh" aerosols as described by McCrumb, et al. Finally, it is hoped that arrangements can be accomplished to study the capacity of P. tularensis to survive and to multiply within macrophages as a function of aerosol age. Since entry into and multiplication within macrophages has been demonstrated in the incipency of inhalation tularemia important clues as to just how infectivity of P. tularensis is determined might result from this model.

#### ACKNOWLEDGEMENTS

The encouragement and assistance of many individuals were essential to the conduct of these studies; among them were. Dan Crozier, Colonel, MC; W. D. Tigertt, Colonel, MC; William S. Gochenour, Jr., Lt Colonel, VC; Arthur L. Hogge, Jr., Lt Colonel, VC; Drs. H. T. Eigelsbach and J. V. Jemski, Mr. E. K. Wolfe and Earl Bachtell. These studies were made possible by the loyal group of Seventh Day Adventist soldiers who volunteered as subjects.

## REVIEW OF TULAREMIA

### STUDIES ON TULAREMIA VACCINE 1960-1962

Fred R. McCrumb, M.D.\*

Earlier studies at the University of Maryland and elsewhere revealed the superior immunizing capacity of the live, attenuated tularemia vaccine developed by Eigelsbach when compared with the cell wall antigen of Larson and inactivated vaccine of the Foshay type. Single exposure to attenuated vaccine resulted in a measurable degree of resistance to aerogenic challenge of 300 to 2,000 virulent Pasteurella tularensis cells. Resistance to a respiratory challenge of 23,000 virulent organisms could not be clearly demonstrated. This experience also revealed the consistency of acute febrile illness with or without pneumonitis following respiratory exposure of control subjects presumably fully susceptible to tularemia. In contrast, vaccinees exhibited varying degrees of resistance which has been described in detail previously. Although immune response to vaccination and challenge has been expressed in terms of bacterial agglutinin titers, quantitative differences in resistance to infection are clearly not correlated with this antibody.

The present studies were designed to elucidate further the protective capacity of attenuated vaccine with and without booster immunization, assess the efficacy of attenuated vaccine administered by the respiratory route and define the infectivity of large particle aerosols of P. tularensis.

### METHODS

Throughout these studies, live attenuated vaccine of Eigelsbach (LVS) has been employed as the immunizing antigen. The virulent organism employed as booster antigen and respiratory challenge was the Schu-S4 strain of P. tularensis. Methods of administering vaccine, challenging vaccinees and controls and studying induced disease in volunteers have been described previously (See Report to Commission on Epidemiological Survey, 1959).

### RESULTS

In July, 1960, 50 volunteers received attenuated vaccine by acupuncture. These vaccinees experienced reaction rates and immune responses of the order described previously. This group of volunteers was held for respiratory challenge approximately 12 months following primary vaccination. A second group of 80 volunteers was immunized by acupuncture in March, 1961. The first challenge studies were carried out in September, 1961.

Attempts to measure duration of immunity imparted by LVS and augment immunity by booster with virulent organisms or revaccination with LVS are

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summarized in Table I. In all, 44 vaccinees were exposed to an aerogenic challenge of approximately 25,000 virulent organisms. Although in some instances antibody levels were augmented by either virulent or attenuated booster, there was not a significant change in the degree of resistance afforded against this vigorous a challenge. The over-all degree of protection was less than 50%. As will be noted subsequently, intradermal (ID) booster with virulent organisms was contained by vaccinees who had received LVS by acupuncture some months previously. Two controls who failed to develop any evidence of infection following inhalation of 25,000 organisms have been studied further and will be discussed in detail in a later report. Suffice it to say at this point that individual host variation (natural resistance) may be a significant factor in the few control failures encountered in these studies.

TABLE I. ATTENUATED TULAREMIA VACCINE INDUCED RESISTANCE TO AEROGENIC CHALLENGE WITH VIRULENT ORGANISMS.

GROUP	IMMUNIZATION HISTORY		RESPONSE TO CHALLENGE WITH VIRULENT <u>P. TULARENSIS</u> (25,000 cell dose)						
	Primary Vaccine	Booster	Interval (Months)	No.	Neg.	Mild	Treated	Protection	Controls
I	LVS	-	14	20	1	8	11	45%	4/6
			10	8	2	-	6	25%	5/5
II	LVS	Schu-S4 (2,500 ID)	4						
		Schu-S4 (8,200 ID)	4	7	3	0	4	57%	5/5
III	LVS	LVS	4	9	4	0	5	44%	5/5
TOTALS				44	10	8	26	41%	9/11

Immunization with Virulent Organisms and Chemoprophylaxis. Fortuitous administration of streptomycin to a control subject in an earlier study resulted in the development of high levels of agglutinins in the absence of significant illness. The possibility that a proper combination of virulent organisms and suppressive antibiotic might result regularly in an immune reaction was suggested. Accordingly, 20 volunteers received virulent organisms (Schu-S4) ID followed by a single dose of 0.5 or 2.0 gm of streptomycin. Inoculations of 2,500 and 10,000 virulent organisms respectively were not controlled by the quantities of antibiotic employed

and 18 of the 20 volunteers developed unmodified disease (Table II). Some degree of suppression was accomplished as evidenced by prolonged incubation periods of 7 to 10 days. However, it may be concluded that under the conditions of this experience, uncomplicated immunization of the man with drug-suppressed virulent P. tularensis was not possible.

TABLE II. IMMUNIZATION WITH VIRULENT P. TULARENSIS, SCHU-S4, AND CHEMOPROPHYLAXIS.

ID VACCINATION		RESPONSE TO STREPTOMYCIN CHEMOPROPHYLAXIS		
Dose	Interval	<u>Drug Dosage</u> (gm)	Relapse <sup>a</sup> /	<u>Incubation</u> (days)
2,500	30 minutes	0.5	9/10	7-10
10,000	24 hours	2.0	9/10	7- 9

a. Number of volunteers developing unmodified disease/number treated with streptomycin.

Aerogenic Immunization with LVS. Immunization of man against tularemia with attenuated vaccine administered by the respiratory route was first studied at Fort Detrick. In this group of 54 volunteers it was demonstrated that inhaled doses of 1,500 to 30,000 viable organisms uniformly resulted in the development of circulating agglutinins. Smaller inocula gave less consistent results (Table III). In March, 1962, 58 volunteers were exposed to

TABLE III. AEROGENIC IMMUNIZATION WITH LVS TULAREMIA VACCINE.

STUDY GROUP	<u>DOSE INHALED</u> (organisms)	NUMBER VACCINATED	<u>SEROCONVERSION<sup>a</sup></u> (%)
Fort Detrick	200- 1,200	24	25-50
	1,500-30,000	30	100
University of Maryland	-	58	84
	17,000-33,000	33	65

a. Percentage of vaccinees developing serum agglutinins following exposure to vaccine aerosol.

a vaccine aerosol of unknown viable population. In May, 1962, 33 additional volunteers received 17,000 to 30,000 organisms by the respiratory route. In both instances, a small particle, dynamic aerosol was employed. Sero-conversion rates varied from 65 to 84% even at this level of exposure. The discrepancy between this experience and studies conducted at Fort Detrick remain to be explained. Conditions under which the aerosols were produced may be a major factor in these contradictory results since vaccine was aerosolized at Fort Detrick under different circumstances.

Resistance to respiratory challenge following aerogenic immunization was found to be fairly good in vaccinees who developed agglutinins (Table IV).

TABLE IV. RESISTANCE TO RESPIRATORY CHALLENGE WITH VIRULENT P. TULARENSIS FOLLOWING AEROGENIC VACCINATION WITH LVS.

VACCINEES		CHALLENGE DOSE				TOTALS
		500	1,700	14,000	25,000	
Serologically Positive	Number	9	5	14	12	40
	Negative	9	3	5	9	26
	Mild	0	1	7	0	8
	Treated	0	1	2	3	6
	Protection	100%	80%	86%	75%	
Serologically Negative	Number	0	0	1	0	1
	Negative	0	3	1	0	4
	Treated	2	2	3	1	8
	Protection	0%	60%	40%	0%	
	Controls <sup>a/</sup>	5/5	5/5	5/5	4/5	19/20

a. Number volunteers requiring therapy for unmodified disease/number challenged.

Although the numbers are limited, results of this study compare favorably with results of percutaneous vaccination, there being protection of 75 to 100% of aerogenic vaccinees even when a large respiratory challenge was employed. Of some interest is the apparent partial resistance among 5 vaccinees who failed to develop detectable agglutinins. Resistance of one control subject to a respiratory challenge of 25,000 organisms is noted again.

Vaccine-Induced Resistance to Intradermal Challenge. Although primary consideration has been given to the problem of inducing immunity to airborne *P. tularensis*, it was deemed worthwhile to attempt the measurement of resistance to a parenteral challenge. Accordingly, small groups of vaccinees immunized by acupuncture or the respiratory route were challenged ID with graded doses of Schu-S4. Results of this study indicate that a high degree of resistance to 10,000 or less virulent organisms administered ID is induced by LVS (Table V). The extent to which LVS might protect against larger challenges can only be answered by additional study. However, it would appear that some vaccinees are capable of resisting an ID challenge of 100,000 virulent organisms.

TABLE V. ATTENUATED TULAREMIA VACCINE-INDUCED RESISTANCE TO ID CHALLENGE WITH VIRULENT ORGANISMS.

PRIMARY VACCINATION WITH LVS	INTERVAL (months)	Dose	RESPONSE TO VIRULENT ID CHALLENGE				
			Neg.	Mild	Treated	Protection (%)	Controls
Acupuncture	6	2,500	9	0	1	90	10/10 <sup>a/</sup>
	6	8,200	7	0	1	88	1/1
	14	100,000	2	0	1	67	-
Aerogenic	2	10,000	7	0	0	100	2/2
		100,000	1	0	2	33	-

a. Number volunteers developing overt disease/number challenged.

Infectivity of Large Particle Aerosols of *P. tularensis*. Several attempts have been made to define the human infective dose of *P. tularensis* suspended in an aerosol composed of 8 to 10  $\mu$  particles. Marked variation in the results have posed more questions than have been answered by this experience. It may be seen that infections have been induced with varying percentages of volunteers with great variations in infecting doses (Table VI). These divergent results are best explained on the basis of technical aberrations. Further study is required to resolve these divergent results. It is of interest to note that abdominal complaints were common among this group suggesting that the route of infection in some subjects may have been the gastrointestinal tract. Further analysis of clinical data will be presented in subsequent reports.

TABLE VI. INFECTIVITY OF P. TULARENSIS IN LARGE PARTICLE AEROSOL.

DATE	PARTICLE SIZE ( $\mu$ )	DOSE	OVERT DISEASE
May, 1962	8.8	26,000-53,000	1/7
	10.5	36,000-81,000	5/6
August, 1962		0	0/7
	8.3	9- 199	6/6
		842-1,124	2/2
August, 1962	8.4	525-2,000	0/8

a. Number volunteers developing overt disease/number challenged.

#### SUMMARY

Additional studies have established the limitations of live, attenuated tularemia vaccine (LVS) in the prevention of aerogenic infection in man. Thus, it appears that resistance to a respiratory challenge of 25,000 virulent organisms cannot be induced in more than 50% of vaccinees who receive a primary percutaneous immunization. Further, revaccination with LVS or boosting with virulent organisms does not augment significantly the degree of resistance afforded by primary vaccination. Studies in which this immunity is compared with that associated with recovery from unmodified disease are in progress.

Aerogenic immunization with LVS resulted in a degree of resistance to virulent challenge comparable to immunity induced by percutaneous vaccination, however, take rates among vaccinees were significantly less than 100% in one group of volunteers receiving large doses of aerosolized vaccine. It would appear that standardization of aerogenic vaccinations with LVS will require additional study.

Attempts to immunize man by suppressing parenterally administered virulent P. tularensis with streptomycin have failed. It is unlikely that single doses of streptomycin will result in the control of an inapparent immunizing infection with this organism.

Studies on the infectivity for man of large particle aerosols containing virulent P. tularensis have yielded contradictory data. Further work on this problem will be required to resolve the question of infectivity of large particle aerosols.

## REVIEW OF TYPHOID FEVER

### VOLUNTEER STUDIES

Richard B. Hornick, M.D.\*

Typhoid vaccines have been in use since the turn of this century. Numerous field trials have been conducted but there is no unanimity of opinion regarding interpretation of the results. Studies of induced typhoid fever in volunteers at the Maryland House of Correction have been designed in an attempt to answer whether various typhoid vaccines and purified antigenic fraction are capable of ameliorating or preventing this disease.

In the course of these experiments, it has been possible to study the pathogenesis of typhoid fever and define attendant pathophysiological alterations. In previous reports to this Commission, results of attempts to establish a reproducible and clinically typical typhoid infection have been presented. In general, a predictable clinical course follows the ingestion of 10 million to 1 billion viable organisms of the Quailles strain of Salmonella typhosa. Ingestion of 100,000 cells have resulted in an identical clinical syndrome but less regularly. The Quailles strain was isolated from a typhoid carrier in 1958. It was obtained from bile draining from a T-tube placed in the patient's common duct following cholecystectomy for cholelithiasis. Aliquots of this culture have been stored at -70 C in skimmed milk. At the time of ingestion, infecting organisms have been transferred in vitro only 4-5 times. Antigenic analysis by Col. S. Gaines of the WRAIR has revealed that the Quailles strain contains about 1/7 the amount of Vi antigen as the Ty2 strain. It has not been possible to produce a non-Vi containing variant of the Quailles strain.

Selected volunteers from the Maryland House of Correction at Jessups, Md., were used in these studies. The infecting inoculum was given in milk, which was gargled several times and then swallowed. The average incubation period has been 6 days. Figure 1 depicts the clinical course of a typical induced infection, although the incubation period is reduced. Coincident with the onset of fever and symptoms was the appearance of bacteremia which persisted for 6 days. Bone marrow cultures correlated well with bacteremia. Quantitative studies revealed the presence of 10 to 50 organisms per ml of blood. The initiation of chloramphenicol therapy has been associated with prompt sterilization of the blood stream in all cases.

Stool cultures yielded infecting organisms during the first 3 days following ingestion. Volunteers did not become ill following a 1000-organisms challenge yet they might have positive stool cultures in the

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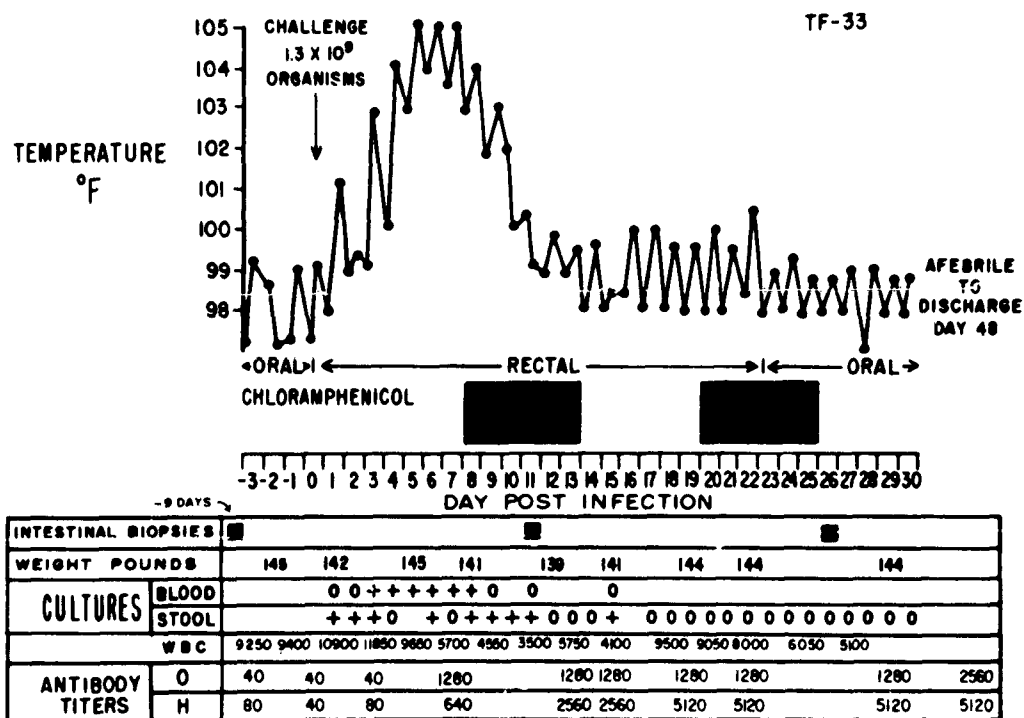


FIGURE 1. CLINICAL COURSE OF A TYPICAL INDUCED TYPHOID FEVER INJECTION.

immediate postingestion period. This suggests that multiplication in the gastrointestinal tract has occurred but not of sufficient magnitude to permit establishment of a clinically overt infection.

Leukopenia was usually demonstrable on the 10th-12th day postinfection. Agglutinin antibody was demonstrable during the second week following ingestion.

Serum complement and properdin levels have been measured through the courtesy of Dr. A. Schubart of the Division of Arthritis, Department of Medicine. Complement levels remained stable during the incubation period and oscillated during the febrile stage. When the temperature returned to normal the level of serum complement rose significantly. Properdin levels on the other hand decreased with lysis of fever but then rebounded to almost twice normal levels during the next few days. In one patient with subclinical disease (low grade fever, monoarticular arthritis and positive blood and stool cultures) who required no therapy, there was no measurable alteration in either properdin or complement baseline values. There appears to be a stimulus during the febrile period for the production of complement which exceeds its consumption. Maksimova (1961) reported that complement titers in 150 cases of randomly selected naturally acquired typhoid fever were reduced. Also, Ecker, *et al* in Cleveland had similar results with this infection in 10 patients. In untreated cases or with late treatment, complement is apparently rapidly consumed in the An-Ab reactions as host defenses are stressed and probably lags complement production.

Measurements of the activity of the reticulo-endothelial system (RES) have been done by Dr. Henry Wagner of the Department of Medicine, The Johns Hopkins School of Medicine. The technique adopted for the determination of the activity of the RES was to administer intravenously  $I^{131}$  tagged, heat-denatured human albumin. Measurement of the radioactivity in blood samples drawn at 5 and 20 minutes gave quantitative estimates of the ability of the RES to clear the heated albumin from the blood. Each volunteer acted as his own control as at least 3 baseline clearance studies were made prior to infection. Results showed that cells of the RES maintained their usual state of activity until fever was lysing. At this phase of the disease, there was evidence of a significant increase in RES activity. Similar results were obtained in volunteers infected with Pasteurella tularensis.

Therapy consisted of chloramphenicol given in an interrupted course of 5 days on therapy, a week without, and a final 5 days of therapy; this regimen has been associated with a 16% relapse rate. Early institution of antibiotic therapy does not appear to influence the incidence of relapses but further data are necessary to evaluate this point. Relapse occurred in one man when chloramphenicol was withheld until the 7th day of disease.

Administration of typhoid vaccines to volunteers has been completed. These vaccines have been described in detail in the previous report to the Commission of Epidemiology (April 1962). In summary, four vaccines have been employed. (a) TAB, a standard commercial triple antigen preparation, (b) Vaccine K, (c) Vaccine L and, (d) Vi antigen, administered in various concentrations to volunteers as a single infection. Vaccines K and L are single whole organism preparations which have been inactivated with heat-phenol and acetone respectively. The dosage schedules for TAB, Vaccines K and L have been identical. Each volunteer received three inoculations of 0.5 ml each. The first two were a week apart and the third one month after the second.

One hundred and forty-eight men were enrolled in this phase of the study. The geometric mean O antibody titer in this group was 1:22 with a range of 1:10 to 1:1280. H antibody titer was only slightly higher, 1:32 with a similar range. There were only 13 men with baseline Vi titers of 1:15 or greater in the entire group. One of these was an Egyptian student whose serum titer of 1:480 was the extreme of the Vi antibody range. He had been vaccinated with TAB about one year previously. Attempts to demonstrate a carrier state were unsuccessful.

Serological results following vaccine administration are in agreement with previously published series. Vaccines containing whole organisms produced measurable Vi antibodies in about 2/3 of the recipients. Purified Vi antigen produced a significant rise in titer in 90%. Vi antibody titers following immunization with TAB, Vaccine K or L were lower than those resulting from the administration of purified Vi antigen.

In contrast to these results which occurred following the administration of chemical- and heat-treated organisms are the results obtained in the volunteers infected with viable bacilli. Although the Quail strain contains Vi antigen, presence of Vi hemagglutinating antibodies was detected in only 8 of 54 volunteers infected with this strain. The highest titer occurred in a patient who relapsed when his serum Vi hemagglutination titer was 1:480. These and subsequent data obtained from Vi-antigen-vaccinated men challenged with virulent S. typhosa bacilli do not correlate with the results obtained in rabbits. These animals can be shown to develop high serum Vi titers following immunization with Vi antigen. The presence of these antibodies plus complement results in marked in vitro bactericidal activity against S. typhosa organisms. Volunteers with equally elevated serum hemagglutinin titers had positive blood cultures during their disease. This suggests that the role of Vi antibodies is of little importance in human resistance to typhoid fever. Further studies are in progress to investigate this hypothesis.

Half of the men remaining in the study have received booster inoculations with their respective vaccines. Serological tests are not complete.

Challenge of 6 in the Vi antigen group resulted in clinical disease in 5. Similarly, 8 of 10 who had received either TAB, or Vaccines K or L developed clinical illness, the other 2 had subclinical disease as manifested by myalgia and low grade fever. All of these men received 1 billion viable S. typhosa as the challenge inoculum. Vi vaccinees were challenged 7 weeks postimmunization and all the other vaccinees were challenged 9 weeks after the third dose of vaccine.

Additional studies to delineate the ID<sub>50</sub> of the Quailes strain for man are in progress. Future studies are planned in which attempts will be made to alter the susceptibility of the host to typhoid fever by using antibiotics, antacids and cathartics before challenge.

## REVIEW OF TYPHOID FEVER

### THE ROLE OF ENDOTOXIN IN TYPHOID FEVER AND TULAREMIA IN MAN

Sheldon E. Greisman, M.D.\*

One major objection to ascribing to endotoxin any significant role in the pathogenesis of typhoid fever or tularemia is based upon the phenomenon of tolerance. Thus tolerance to the pyrogenic and toxic activities of endotoxin is acquired rapidly in man following single daily intravenous injections, and several investigators have therefore concluded that endotoxin cannot play any important role in those infections in which fever is sustained.

Studies currently in progress indicate that this thesis is untenable. Two volunteers have been rendered tolerant to the pyrogenic and toxic effects of Salmonella typhosa endotoxin by gradually increasing increments administered as single daily intravenous quantities. After 3 weeks, these subjects could tolerate an intravenous injection of 2.5  $\mu$ g of S. typhosa endotoxin with no reaction, whereas 0.5  $\mu$ g of the same material initially induced severe toxic and pyrogenic reactions. These subjects were challenged with viable S. typhosa by the oral route; despite their tolerance and elevated O serum agglutinin titers, overt illness developed as in untreated volunteers. On the first day of clinical illness these subjects were tested with 0.5  $\mu$ g specific endotoxin. In both cases, marked febrile and toxic reactions ensued associated with prostration, necessitating the administration of hydrocortisone in one subject. Repeat testing with 0.5  $\mu$ g endotoxin during illness and early convalescence (36 hours) evoked similar severe reactions. Later in convalescence (6th and 18th afebrile days) minimal pyrogenic reactions were evoked by 0.5  $\mu$ g endotoxin and subjective discomfort was virtually absent.

An entirely similar reaction pattern was observed in 3 additional volunteers rendered tolerant to S. typhosa endotoxin and infected with Pasteurella tularensis. In each case, after endotoxin tolerance was established by daily single intravenous injections for 2-4 weeks, challenge with  $\frac{1}{4}$  the dose of endotoxin led to extremely severe pyrogenic and subjective reactions during overt illness and early convalescence. No loss of tolerance was noted during the incubation phase. Tolerance began to return after several days of convalescence.

In all subjects, despite preceding tolerance, the reactions to endotoxin during overt illness with typhoid fever and tularemia were distinctly more severe than the initial "virgin" reaction to the same quantity of endotoxin.

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Parallel studies conducted in 4 volunteers given sandfly fever revealed that tolerance to S. typhosa endotoxin was maintained during illness, and full doses of endotoxin could be tolerated with no discomfort or increase in fever index.

These data indicate that during typhoid fever and tularemia in man, the mechanisms responsible for endotoxin tolerance cease to operate effectively; indeed, despite preceding tolerance, man now becomes more reactive to endotoxin than during his initial "virgin" contact. This paralysis of the endotoxin tolerance mechanism does not appear attributable to fever or infection per se as judged by the sandfly fever studies.

Studies are now in progress to define the mechanism for loss of tolerance during typhoid fever and tularemia. Preliminary studies conducted in conjunction with Dr. Henry Wagner of The Johns Hopkins Hospital indicate that endotoxin tolerance in man is not associated with acceleration of reticulo-endothelial system (RES) activity as measured by clearance of  $I^{131}$  tagged heat-denatured human serum albumin. This suggests that endotoxin tolerance in man can develop without any general increase in phagocytic activity of the RES. Our current working hypothesis is that humoral factors are elaborated during the development of tolerance which specifically permit more rapid uptake of endotoxin by the RES; these humoral protective factors are "tied up" by endotoxin released during typhoid fever and tularemia so that additional exogenous endotoxin remains free to induce its toxic effects at the target sites. This hypothesis is supported by the results of reversal of tolerance in man by administration of divided doses of endotoxin spaced 2 hours apart, final confirmation awaits passive transfer and endotoxin clearance studies, the former of which is currently in progress.

## REVIEW OF VENEZUELAN EQUINE ENCEPHALOMYELITIS

### ATTENUATED STRAIN OF VIRUS

Robert W. McKinney, Major, MSC\*

Experience with the use of the attenuated strain of Venezuelan equine encephalomyelitis (VEE) virus, while more than satisfactory from the standpoint of eliciting immunity, was offset by the reactions which occurred following its administration.

Accordingly, attempts were made to obtain a virus preparation having lesser reactogenic properties. Using 78th passage virus as seed material, the VEE virus was grown in chick fibroblast tissue cultures. From the culture inoculated with the highest dilution in which plaques appeared in significant number, 12 plaques were picked and individually passaged in secondary fetal guinea pig heart cell cultures. At this time human serum albumin (USP) was substituted for the bovine serum albumin, thus making it possible to maintain protein concentration throughout the range of dilutions employed without the problem of sensitizing man to a foreign protein.

The virus replicated from each plaque was titrated in mice by the intracerebral (IC) route in dilutions ranging from  $10^{-1}$  through  $10^{-6}$  using 6 animals per dilution. Death was used as an index of residual virulence.

Of the 12 plaques, 9 yielded virus which killed 3-23% of mice infected independent of dose. The virus suspension replicated from the other 3 plaques each had a mouse IC immunizing titer of greater than  $10^{-5.4}$ .

The virus obtained from the culture of one of these 3 plaques was employed as seed for preparing a single lot of material. The virus was propagated in fetal guinea pig heart cells maintained under Hank's balanced salt solution plus 0.5% human serum albumin.

Titration of the virus in guinea pigs gave evidence that this material differed from previous preparations in that febrile response was no longer correlated with infection and serologic response. In addition, infectivity for the mouse by either the IC or intraperitoneal (IP) route was reduced.

At this time an additional lot, 81st passage level virus, was prepared. The IP immunizing titer of this material in guinea pigs was  $10^{-6.5}$ ; again, not all animals infected developed fever while all which responded serologically resisted challenge with approximately 1,000 LD<sub>50</sub> Trinidad virus.

Since the results obtained in animals indicated a reduction in reactivity of the virus as compared with that previously employed, 7 individuals, none of whom were known to have had experience with the

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virus were administered approximately 700 guinea pig immunizing doses. Two of the 7 developed mild upper respiratory infection signs and symptoms of short duration. One individual failed to develop antibody; however, on re-inoculation a response was obtained without reactions.

The reactions which occurred were significantly less severe than those previously observed. Human trials with the virus were extended to a partial titration. Eleven individuals were divided into groups of 4, 3 and 4, and were inoculated with 0.5 ml doses containing  $6 \times 10^4$ ,  $6 \times 10^3$ , and  $6 \times 10^2$  guinea pig immunizing units of virus respectively (Table I). Reactions were graded subjectively, and none of the individuals inoculated nor the examining physician were aware of the dose inoculated. The reactions were graded 4+ - disabled for duty; 3+ - severe but not disabling; 2+ - moderate; 1+ - present but insignificant, and negative - none. The reactions ranged from minimal headaches plus a feverish feeling without temperature increase (TB) to one (WC) who complained of mild chilliness, myalgia, and moderate headache. Oral temperature of WC at the time of complaint was 102 F. While reactions did occur, the most severe was felt to be of significantly lesser intensity than those previously observed. The individuals who failed to develop antibody are of interest. They had received yellow fever vaccine 3 days earlier, it would appear reasonable that they failed to respond as a result of interference between the 2 viruses. On re-inoculation all 3 responded with the development of antibody and only one had any clinical reaction.

Concurrently with the foregoing study the same quantities of virus were inoculated into 6 burros (2 at each dose level) and 5 rhesus monkeys (2 each receiving 60,000 and 6,000 infective doses respectively, and one 600). None of the monkeys developed fever or other signs of infection during the 12 days following inoculation; however, all developed significant hemagglutination-inhibition (HI) and complement-fixation (CF) antibody titers. Three of the 6 burros became febrile (Figure 1); the febrile period was short-lived and none of the animals exhibited any clinical signs. The febrile pattern differed from that previously seen in animals infected with earlier preparations, wherein inoculation of a comparable dose had resulted in a fever pattern which resembled that seen with Trinidad strain infection in this species. Again, all 6 animals responded serologically and on subsequent challenge were resistant to infection with virulent virus.

Since completion of this study, additional persons have been given the virus. Some had received killed vaccine while others were without prior experience. The results obtained with the former group are presented in Table II. While it would appear that the killed vaccine was effective, and indeed did prevent infection with the virus, evidence has been accumulated that at least some individuals administered the killed vaccine have incurred subclinical infection. No reactions were observed in this group; and in 20 other individuals without prior experience, only 2 had mild reactions.

TABLE I. ANTIBODY RESPONSES IN MAN INOCULATED SUBCUTANEOUSLY WITH ATTENUATED VEE VIRUS.

INOCULUM DILUTION (GPID) <sup>a</sup> /	CASE	SYMPTOMS	ANTIBODY TITERS				
			HI <sup>b</sup> /		CF <sup>c</sup> /		SNI <sup>d</sup> /
			14	25	14	25	
10 <sup>-1</sup> (6 x 10 <sup>4</sup> )	TB	2+	80	80	-	8	2.5 x 10 <sup>3</sup>
	MW	2+	20	80	-	4	2.3 x 10 <sup>1</sup>
	WC	3+	160	160	2	16	1.9 x 10 <sup>3</sup>
	SF	3+	640	320	2	16	1.2 x 10 <sup>4</sup>
10 <sup>-2</sup> (6 x 10 <sup>3</sup> )	FN	3+	40	80	2	8	≥7.0 x 10 <sup>2</sup>
	FM	-	80	160	-	8	5.0 x 10 <sup>1</sup>
	JS <sup>e</sup> /	-	-	-	-	-	ND <sup>f</sup> /
10 <sup>-3</sup> (6 x 10 <sup>2</sup> )	LR	-	80	160	2	4	1.6 x 10 <sup>1</sup>
	HE	-	40	640	-	64	>3.9 x 10 <sup>3</sup>
	JB <sup>e</sup> /	2+	-	-	-	-	ND
	LT <sup>e</sup> /	3+	-	-	-	-	ND

a. GPID - guinea pig immunizing units.

b. Hemagglutination-inhibition reciprocal titer; - equals <10.

c. Complement-fixation reciprocal titer; - equals <2.

d. SNI - serum neutralization index in mice; 0- and 25-day sera.

e. Yellow fever 3 days earlier.

f. ND - not done.

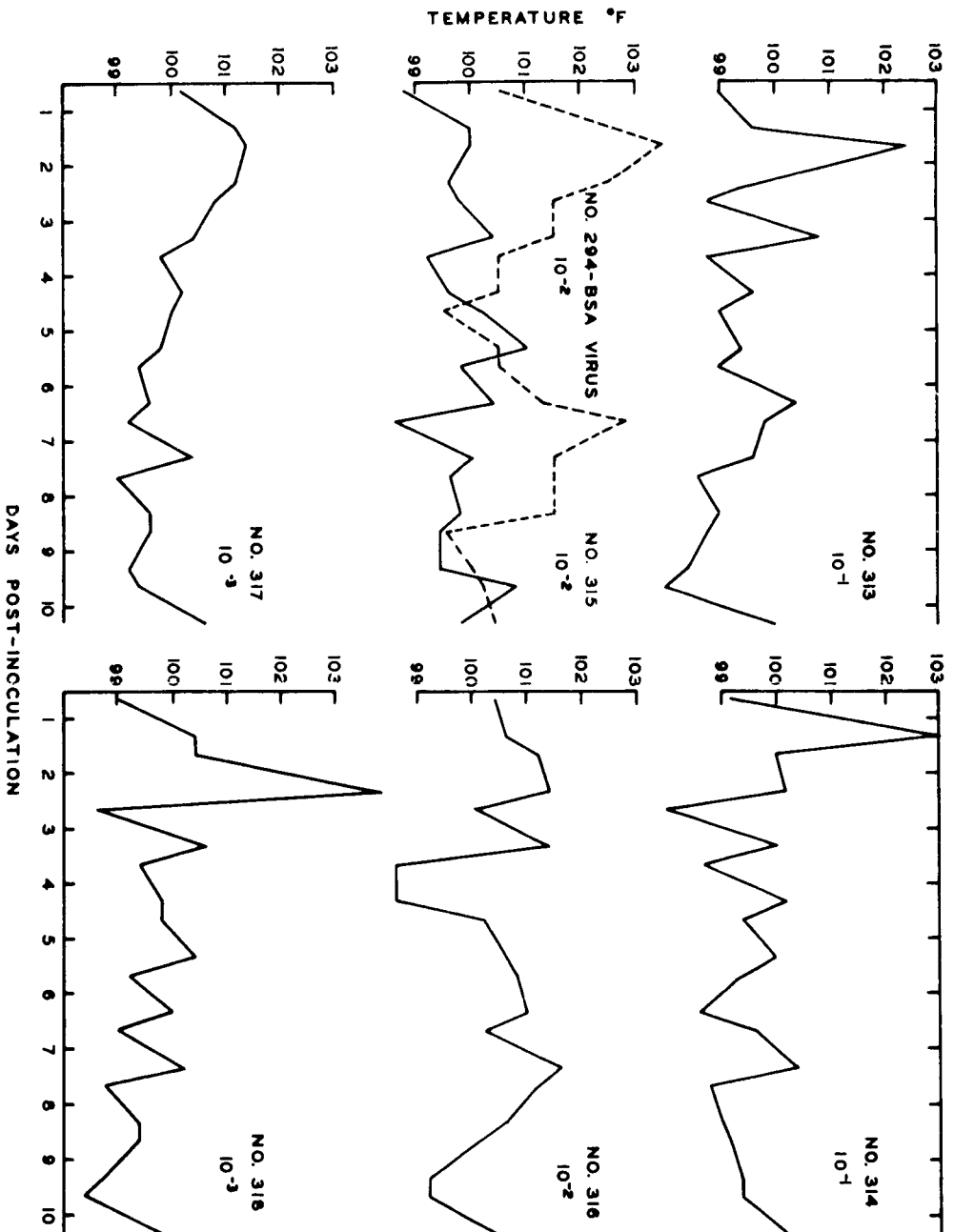


FIGURE 1. FEBRILE RESPONSES IN BURROS INOCULATED  
WITH ATTENUATED VEE (TC 81 HSA) VIRUS (LOT 2-4)  
1.0 ML OF VARYING DILUTIONS

TABLE II. HI<sup>a/</sup> ANTIBODY RESPONSES IN PERSONS ADMINISTERED INACTIVATED VACCINE AND ATTENUATED VEE VIRUS.

PREVACCINE TITER <sup>b/</sup>	NUMBER	NUMBER BY FOLD-INCREASE IN HI ANTIBODY TITER									
		NC/2 <sup>c/</sup>	4	8	16	32	64	128	256	512	1024
<10	9	-	1	1	3		1	1		1	1
10	7	3	1	2			1				
20	10	8	-	2							
40	13	10	2	1							
80	9	9									
160	8	8									
320	0	-									
640	2	2									
1280											
2560	1	1									

a. HI - Hemagglutination-inhibition.

b. Reciprocal of titer.

c. NC/2 - No change or 2-fold increase.

Berge, et al<sup>1/</sup> found that the attenuated virus underwent reversion on serial IC passage in the mouse. Because the plaque-derived virus appeared less infective for the mouse, these studies were repeated. Again, lethality for the mouse was regained, the rate of reversion being the same as in the earlier studies.

The fact that the virus undergoes reversion on serial IC passage in the mouse plus the viremic state in individuals infected with the virus prompted consideration of the question of possible transmission by mosquitoes and the consequences thereof. Studies directed to this question are in progress and include feeding mosquitoes on infected guinea pigs, infection by hanging drop technique and by intrathoracic inoculation plus direct feeding on man. It is not planned to attempt serial transmission by mosquitoes in man but restrict such studies to laboratory animals. Aedes triseriatus is being employed in this work.

Using Trinidad strain virus for backchallenge of animals, 100% of guinea pigs which develop HI antibody have been found to be immune to a standard challenge dose of 1,000 LD<sub>50</sub>. In addition, guinea pigs and mice infected with the attenuated virus have survived IP challenge with up to  $1 \times 10^9$  lethal doses of any of the following strains: Trinidad, Colombian, 2 Panama strains and a strain isolated from a mosquito pool collected recently in Trinidad. Mice similarly immunized and challenged by the IC route resisted a dose of  $10^7$  mouse IC lethal doses of virus.

At the present time the attenuated VEE virus is under contract development. A large pool of 92nd passage virus has been prepared, and at present, all lots of vaccine will be 93rd passage. The results of assays indicate no apparent changes occurred during the 12 passages from the original plaque. The current procedure for immunization of "at risk" personnel is the subcutaneous injection of 0.5 ml of virus suspension containing between 500 to 1,000 guinea pig IP immunizing doses of virus.

#### REFERENCE

1. Berge, T. O., Banks, I. S., and Tigertt, W. D.. "Attenuation of Venezuelan Equine Encephalomyelitis Virus by in vitro Cultivation in Guinea-Pig Heart Cells," Amer. J. Hyg. 73 209-218, 1961.

## REVIEW OF ANTHRAX

### STUDIES ON ANTHRAX TOXIN

Martha K. Ward, Captain, USPHS\*

Since about 1945 it has been clearly and repeatedly demonstrated, first by Cromartie and his co-workers at Fort Detrick, then by the Porton group, that extracts of anthrax lesions and plasma of untreated animals dying of anthrax contain a specific toxic substance capable of producing edematous lesions in the skin of rabbits and guinea pigs and death upon intravenous injection in relatively large amounts into mice and guinea pigs.

More recently, the presence of toxic substances with the same kind of biological activity have been demonstrated in in vitro cultures of Bacillus anthracis, both by the Porton group and by Thorne, Molnar and Strange at Fort Detrick. These latter workers, who were able to produce in vitro toxin in the absence of serum in the medium, also very ingeniously demonstrated that the in vitro toxin could be separated into 2 components, one of which was very closely related to or identical with so-called protective antigen.

That the anthrax organism does produce, both in vitro and in vivo, diffusible poisons detectable by certain biological and serological procedures is therefore a fact which cannot be disputed.

As stated earlier, Thorne and his group were able to separate in vitro toxin into at least 2 components which were nontoxic separately, but which when appropriately recombined exhibited toxic activity.

Smith and the Porton group had demonstrated earlier similar separation of toxic guinea pig plasma into 2 components by ultracentrifugation procedures.

All of the early work with both in vitro and in vivo toxin indicated that the ability of these materials to produce edema in the skin of guinea pigs and lethal activity in mice were very closely related and inseparable properties. However, within the last year or two the work of Beall, Taylor and Thorne, using rats as test animals, has shown that the component necessary to produce death in the rat may be separated from that needed for production of edema in the skin of the guinea pig. Using a different technique, Stanley and Smith have also demonstrated a third component in in vitro anthrax toxin.

All of the work we have done and most of the work on in vitro toxin in other laboratories has, I believe, been done with modified Casamino acids medium described by Thorne and Belton and with the avirulent Weybridge strain

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of anthrax. I believe that I voice the opinion of most people when I say that the only consistent observation is that of variability. Variations in titers and toxicity occur from batch to batch and from flask to flask within a batch. This variability cannot be correlated completely with final yield of cells in our experience and I believe in the experience of others. This again demonstrates that certain conditions may affect growth and others, primarily the production of toxin.

Some more recent work in our laboratory by Commander Gaspar would suggest that some of this variability might be overcome by doubling the concentration of Casamino acids in the medium and perhaps also by substituting KCl for the phosphate in the medium.

The major production problems now to be dealt with are those of concentration and purification of large enough quantities of reproducible lots of material for physiological and physicochemical studies.

Most of the work on concentration and purification of toxin done by other laboratories has been on the 3 known components of toxin after they have been separated from each other by glass filtration or column chromatography.

In view of the facts that the biological activity of recombinations of these components appears to be related to the proportions of each present in various mixtures and that little is known about the actual proportions of each which exist in the original starting material as produced by the organisms, we have been attempting to find methods to concentrate the toxic activity of whole culture filtrates and avoid procedures known to split toxin into its component parts.

Initial work indicates some success in this regard. By using Millipore filters for removal of the organisms, concentration by Carbowax with subsequent dialysis and fractional precipitation with ethyl alcohol, we have been able to concentrate the toxic activity of the starting material about 100-fold. The methods used however do not give clean cuts of activity and even with this much concentration the final preparation contains only about 160 mg%/nitrogen, so further attempts to simplify the procedure and achieve greater concentration of the material are in progress. We are of course still trying to obtain initial yields of crude toxin but so far all of these attempts have failed. The best yields in crude culture filtrates have been about 32 units/ml. It has been calculated that 1 unit of activity represents about 0.5  $\mu$ g of active protein so that even with a 100-fold concentration of material we don't have much to work with for physical or chemical analysis when we can handle only about a maximum of 10 L per batch. However, there is some cause for hope that better concentration of the material will soon be achieved and that we can increase the quantity of material available for physiological and physicochemical studies.

There are also problems involved in the assay of this material. The guinea pig skin edema assay which seems to be quite sensitive and reproducible unfortunately detects the presence of only 2 of the 3 known components of toxin. Lethality tests in mice and rats require relatively large amounts of material and in our hands at least do not give consistently reproducible results. Furthermore, the lethality tests also may indicate the presence of only 2 components of toxin, one of which is different from that measured in the guinea pig skin assay. Consequently both methods must be used at each step in the concentration and purification procedures to have any assurance that one or another component has not been selectively destroyed in the processing.

#### In vivo Toxin:

In the past 6 months studies designed primarily to examine the efficacy of the commercially-produced anthrax protective antigen as an immunizing agent against parenteral challenge in guinea pigs and respiratory challenge in monkeys provided us the opportunity to make some observations on in vivo-produced anthrax toxin.

Plasmas were collected from all moribund animals in the immunized and control groups. They were processed in the manner described by the Porton group, filtered through Millipore filters to remove bacteria and kept at 0 C or freeze-dried until examined.

These plasmas were examined for toxic activity by animal assays and for the presence of antigens and antibody by agar diffusion techniques. In addition, Colonel Gray's laboratory has determined electrophoretic patterns on all of the samples available in sufficient quantity for these studies, using standard paper electrophoresis techniques.

Several points of interest have become apparent as the results of these studies:

1. Evidence of toxin can only very rarely be detected in terminally obtained plasmas of immunized animals. Whenever evidence of toxicity was observed in immunized animals it was present in low titer and in all cases it was found only in animals dying prior to about 140 hours.
2. Toxin in relatively high titer was found in plasmas of all control animals with the exception of 2 monkeys that died after 140 hours. In general as survival time increased toxin titers decreased.
3. Many of the terminal specimens, particularly those from control animals, contained antigens which did not identify in agar diffusion plates with lines present in in vitro-produced toxin.
4. Relatively high titers of neutralizing antibody as measured by both in vivo and in vitro methods were found in the terminal plasmas of the vast majority of immunized animals.

5. The presence and titer of antibody in plasmas appeared to be related both to prechallenge antibody titer and to survival time.

When prechallenge antibody titer was high, titers in moribund plasmas were either the same or somewhat lower than at time of challenge. The longer the animal survived the greater the decrease in titer. On the other hand in groups of animals with lower prechallenge titers, the moribund plasma antibody titers increased markedly with survival time. In other words there appeared to be a booster effect in those animals that survived more than about 120 hours.

The results of the electrophoresis studies on guinea pig plasmas proved interesting in that there was a consistently observed, marked increase in the  $\alpha_2$  globulin fraction. This increase in both absolute value and relative percentage was greatest in control animals, less in animals receiving only 1 dose of antigen and least in animals receiving 3 doses of antigen. There was also a decrease in the albumin fraction, the greatest drop again occurring in the control animals but in this instance there was little difference between the 2 immunized groups.

Such increases in  $\alpha_2$  globulin have of course been reported in other diseases, but this striking rise of 100% or more in many instances above controls and in such a short period of time after infection appears to be unusual. It would be interesting to determine how rapidly any significant change in  $\alpha_2$  globulin occurs in the guinea pig and in other experimental animals to see if such a measurement could be used as an indication of infection prior to any signs of clinical illness.

It is difficult to assess the significance of some of these observations in terms of the role that toxin, as we now define and detect it, may play in the pathogenesis of anthrax infections. However, I do think that they raise a number of interesting questions which need to be answered.

## REPORT OF PHYSICAL SCIENCES DIVISION

U. S. ARMY MEDICAL UNIT

## LYSINE DEFICIENCY AND HOST RESISTANCE TO ANTHRAX

Irving Gray, Colonel, MSC

The interaction between the state of nutrition and the resistance of a host to infectious disease is well established. The purpose of the studies reported here was to follow the host response to an organism and determine how specific modification of the host might affect this response. The host selected was the rat and the parasite, Bacillus anthracis.

Sprague-Dawley, female, white rats, were weaned, shipped by the supplier and received at our laboratory at 22 days of age. Litter-mates were allocated at random into appropriately sized groups and placed on a predetermined diet. The diets were obtained commercially. The composition of the diets is listed in Table I. The experimental diet (G) was one designed to produce a lysine deficiency in the rats. In this diet, the casein of diet C was replaced by gluten and the caloric balance was maintained by increasing the cornstarch as necessary. In the experiments where the effect of dietary supplements was studied, the desired amino acid, L-lysine (GL), L-methionine (GM), or both (GLM) was added to diet G in an amount to make the specific amino acid concentration equal to that in control diet C. The animals were allowed both food and water ad libitum. The rats were maintained on the diet for at least 30 days.

The challenge organism was the Vollum-1-b (Lot 189) strain of B. anthracis. Spores were heat-shocked 48 hours prior to use by subcutaneous challenge. The challenge dose was  $10^7$  spores. After inoculation, mortality was recorded daily. Inasmuch as it was determined in preliminary experiments that deaths rarely occurred after 6 days, all experiments were discontinued at that time.

Various clinical laboratory determinations were obtained by standard methods except for lysozyme determinations. Rat plasma was used at a 1/40 dilution and the standard was 5 µg/ml. For spleen concentration, the whole organ was homogenized in 10 ml of physiological saline, centrifuged at 1,500 rpm for 30 minutes; 4 ml of the supernatant fluid taken for analysis. For these studies the rats were stunned by a blow to the head and sacrificed by exsanguination.

The activity of the fixed macrophages of the reticulo-endothelial system (RES) was studied using colloidal chromic phosphate- $P^{32}$ . Animals were sacrificed at 1, 3, 5, 7, 9, 11, 13, 15, 20, 25 and 30 minutes following inoculation. The disappearance rates were calculated by the method of least squares. The liver and spleen were removed, weighed, and assayed for radioactivity. In

TABLE I. COMPOSITION OF THE CONTROL (C) AND EXPERIMENTAL (G) DIETS.

NUTRIENT	COMPOSITION %	
	Control Diet (C)	Experimental Diet (G)
Vitamin free casein	27	0
Gluten	0	20
Cornstarch	59	66
Corn oil	10	10
Salt mixture USP XIV	4	4
Vitamin diet fortification mixture gms/100 lbs diet		.

those experiments where 24-hour excretion of  $\text{CrP}^{32}\text{O}_4$  was followed, the rats were placed in appropriate metabolic cages in which the urine and feces were separated and collected in each 24-hour period following the injection.

To follow the fate of injected spores, the washed spores were suspended in phosphate buffer, pH 7.4, to which was added 4.2 ml of a mixture containing 0.7 ml of a solution of 126 mg  $\text{I}_2$  in 100 ml 0.1M KI and 3.5 ml of stock carrier-free  $\text{I}^{131}$  (containing 1-2 mc). The spores remained in this mixture for 15 minutes. The final preparation contained less than 5% unbound  $\text{I}^{131}$ . The spores were injected subcutaneously. The urine was collected from individual animals housed in appropriate metabolic cages.

All radioactivity was measured to an error of 5% or less in a well-type scintillation counter.

Figure 1 illustrates the growth rate of two typical groups of 100 rats while Figure 2 records the appearance of the animals. When these animals were challenged with differing concentrations of B. anthracis spores, a marked difference in the mortality between the two groups of rats became apparent. Table II presents the results of several such experiments. It is apparent that lysine-deficient animals are less resistant to infection at all levels of challenge. It was from these data that the decision was made to use  $10^7$  spores for all subsequent challenges. Table III summarizes the data illustrating the decreased resistance of the lysine-deficient rats to a  $10^7$  spore challenge of B. anthracis. That this change

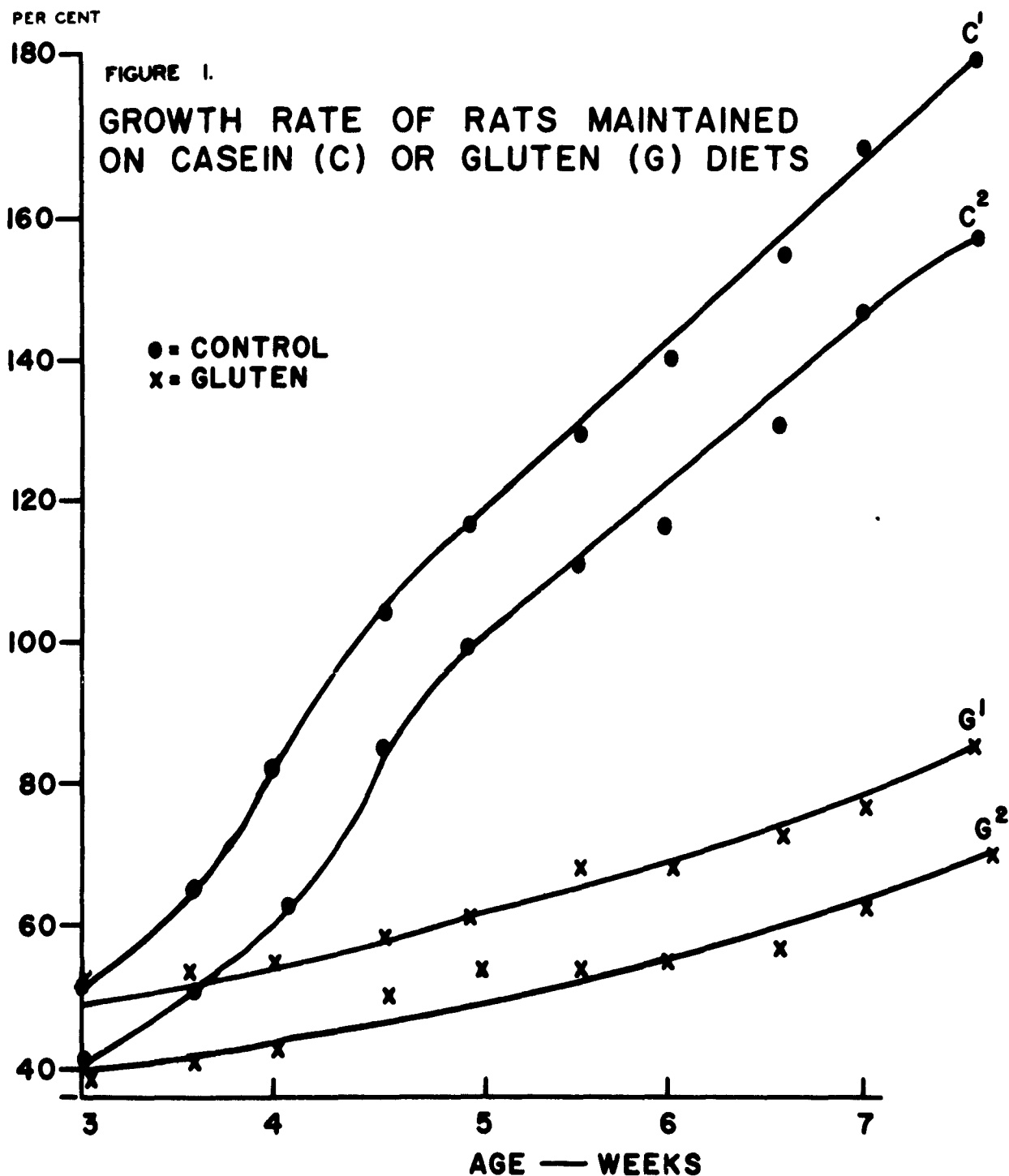




FIGURE 2. APPEARANCE OF SPRAGUE-DAWLEY RATS AFTER BEING FED ON A CASEIN, DIET (C) OR AN EXPERIMENTAL GLUTEN, DIET (G). RATS WEANED AT 21 DAYS OF AGE AND MAINTAINED ON THE DIET FOR 30 DAYS.

TABLE II. RELATION BETWEEN CHALLENGE DOSE, MORTALITY, AND DIET.

DOSE	CUMULATIVE MORTALITY			
	CONTROL		GLUTEN	
	Dead/Total	%	Dead/Total	%
$10^{10}$	22/40	55		
$10^9$	16/40	40	39/40	98
$10^8$	16/40	40	39/40	98
$10^7$	14/40	35	36/40	90
$10^6$	19/50	38	38/50	76
$10^5$			25/50	50

TABLE III. THE EFFECT OF DIET ON MORTALITY OF RATS CHALLENGED WITH  $10^7$  B. ANTHRACIS SPORES.

EXPERIMENT NUMBER	CUMULATIVE MORTALITY	
	CONTROL	GLUTEN
	Dead/Total	Dead/Total
1	7/15	13/15
2	1/15	14/15
3	6/10	9/10
4	5/20	16/20
5	3/20	15/20
6	7/20	15/20
TOTAL	29/100	82/100
%	29	82

in resistance is an acute phenomenon is illustrated in Figure 3. Here it is seen that except for an occasional animal, all deaths in the control group occur by the third day postinoculation. This suggests that the decreased resistance lies in the immediate host reaction to the challenge.

In Table IV the effect of supplementation with the amino acids that are limiting in the gluten diet is reported. When lysine is added to the diet, with or without methionine, the resistance increases over that of totally deficient animals but does not return to that of the control animals. It should be noted that the weight of the experimental animals is similar to that of the controls. This indicates a separation of lack of growth due to amino acid deficiency and resistance to the disease. These findings are similar to those of Schaedler and Dubos who used mice challenged with several different bacterial species.

Table V lists some changes found to be associated with lysine deficiency. The possible importance of the decrease in the number of circulating leukocytes and platelets will be discussed below. The significance of the other findings is more difficult to assess. From the relative serum and urine values of several components, there appears to be a decrease in renal function. However, unpublished data from our laboratory indicate that the endogenous creatinine clearance is unchanged. Since the latter is reported to be not too accurate as a measure of glomerular filtration rate in the rat, other studies are underway to investigate the importance of the renal function changes.

It is apparent that the rate of clearance of colloid from the blood of deficient rats is reduced (Figure 4). Furthermore, when the direct rates of loss as measured by the slope,  $k$ , of disappearance are corrected for the weight of the liver and spleen, the differences between rates,  $\alpha$ , are still evident. Although the loss of radioactive colloid from the blood proceeds at a slower rate, the concentration of colloid in the organs studied is greater in the lysine-deficient animals than in the controls (Figures 5 and 6). Table VI summarizes the results obtained when the excretion of the  $\text{CrP}^{32}\text{O}_4$  is followed in the urine and feces. It is apparent that there is a difference in both the rate of urinary and fecal excretion. There is almost 2 times the radioactivity per gram of feces in the control over that in the deficient animals. On the other hand, the excretion rate in the urine of the gluten animals is greater. This is compatible with the high blood level of  $\text{P}^{32}$  in this group.

Figure 6 summarizes the results on the urinary excretion of breakdown products of the  $\text{I}^{131}$ -labeled spores. The excretion is more rapid and rises to greater values in the control animals. The significant differences occur in the first 3 days following inoculation. After this time, both groups excreted at the same rate.

Lysine deficiency brought about by a gluten diet reduced the resistance of the rat to anthrax. In both the experimental groups and controls, death occurred within 2-3 days postinoculation. This type of response,

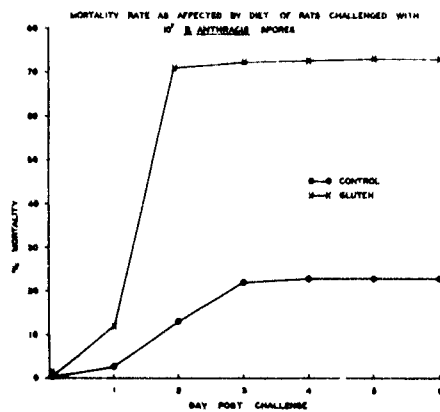


FIGURE 3

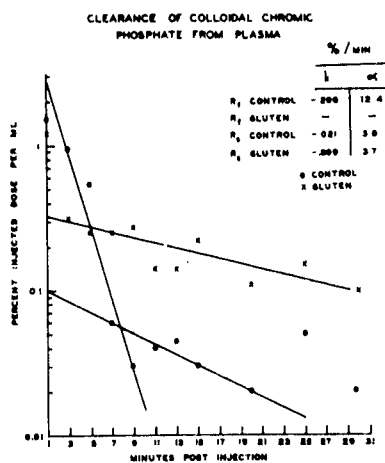


FIGURE 4

TABLE IV. THE EFFECT OF SPECIFIC AMINO ACID SUPPLEMENTATION OF THE DIET ON MORTALITY OF RATS AFTER CHALLENGE WITH  $10^7$  B. ANTHRACIS SPORES.

EXPERIMENT NUMBER	DIET				
	C	G	GL	GM	GLM
4	5/20 <sup>a/</sup>	16/20	8/20	16/20	9/20
5	3/20	15/20	6/10	9/10	7/10
8	3/15	10/15	5/15	8/15	8/15
Total	11/55	41/55	19/45	33/45	24/45
%	20	75	42	73	53
Avg Wt/Rat Gm	165	83	159	84	146

p VALUES BY  $\chi^2$  TEST OF SIGNIFICANCE

DIET	G	GL	GM	GLM	C: Control Diet
					G: Gluten Diet
C	<.001	<.001	<.001	<.001	GL: G + Lysine
G	--	<.001	Not sign.	<.05	GM: G + Methionine
GL	--	--	<.01	Not sign.	GLM: G + Lysine + Methionine
GM	--	--	--	<.01	a/ ~ Number dead/Number Challenged

TABLE V. IN VIVO CHANGES ASSOCIATED WITH LYSINE-DEFICIENCY IN RATS.

DETERMINATION	VALUES		SIGNIFICANCE
	Control Diet	Gluten Diet	
Total blood leukocytes (/mm <sup>3</sup> )	12,700	9,900	p<.01
Total blood platelets (/mm <sup>3</sup> )	478,000	354,000	p<.05
Serum complement (50%units/ml)	148	61	p<.001
Serum lysozyme (μg/ml)	15	11	p<.001
Spleen lysozyme (μg/gm)	27	56	p<.001
Serum total protein (gm%)	6.3	5.1	p<.05
Serum Na <sup>+</sup> (meq/L)	143	138	NSD
Serum K <sup>+</sup> (meq/L)	4.8	6.7	p<.01
Urine pH	5.8	7.3	p<.05
Urine Na <sup>+</sup> (meq/24 hr)	0.66	0.3	---
Urine K <sup>+</sup> (meq/24 hr)	1.12	0.55	---
Urine Cl <sup>-</sup> (meq/24 hr)	3.1	1.4	---
Blood urea N (mg%)	18	31	p<.01
Urea clearance (ml/min)	0.87	0.12	p<.05

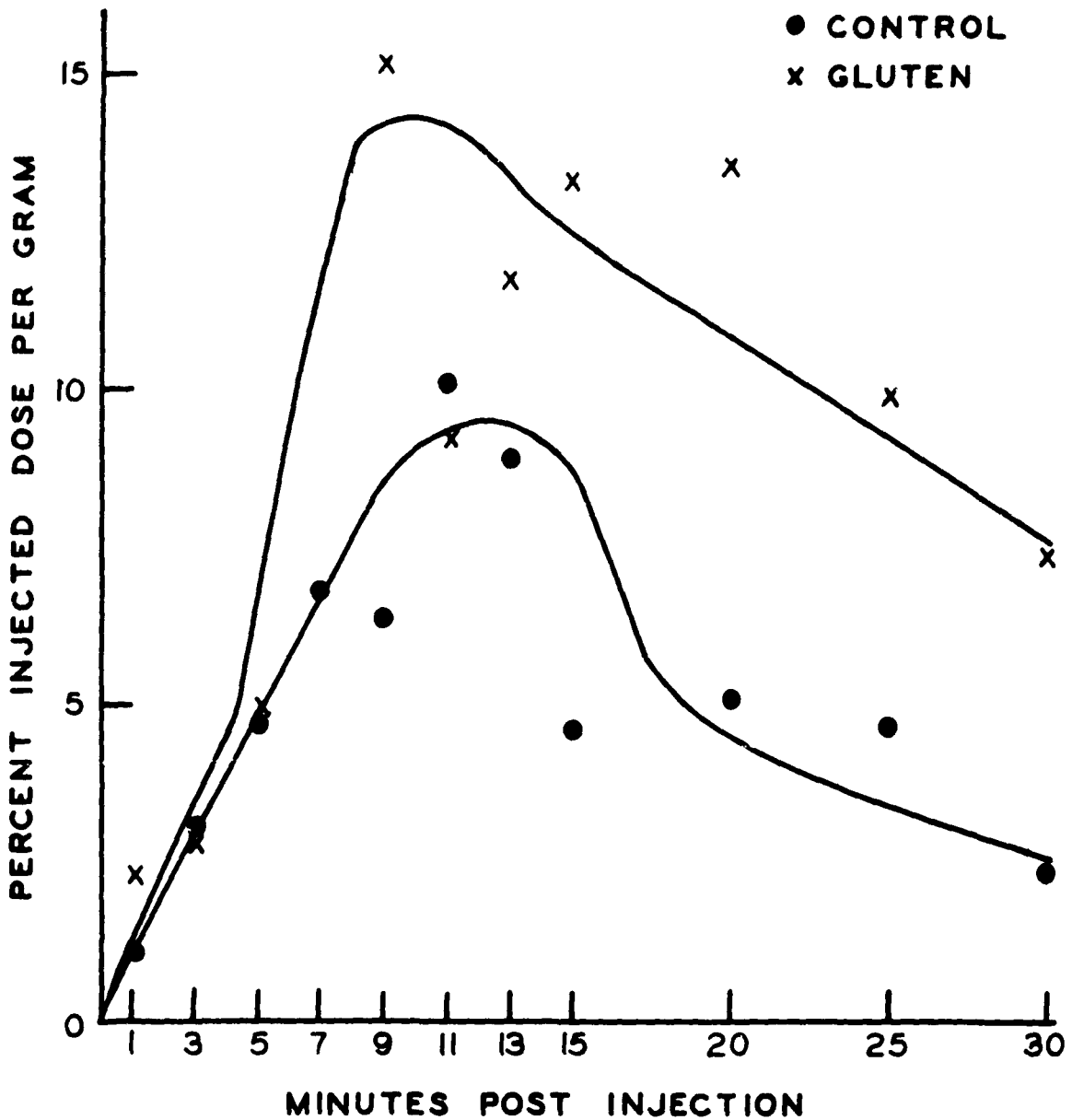


FIGURE 5. RATE OF ACCUMULATION OF COLLOIDAL  $\text{CrP}^{32}\text{O}_4$  IN THE LIVER OF CONTROL AND LYSINE DEFICIENT (GLUTEN DIET) RATS.

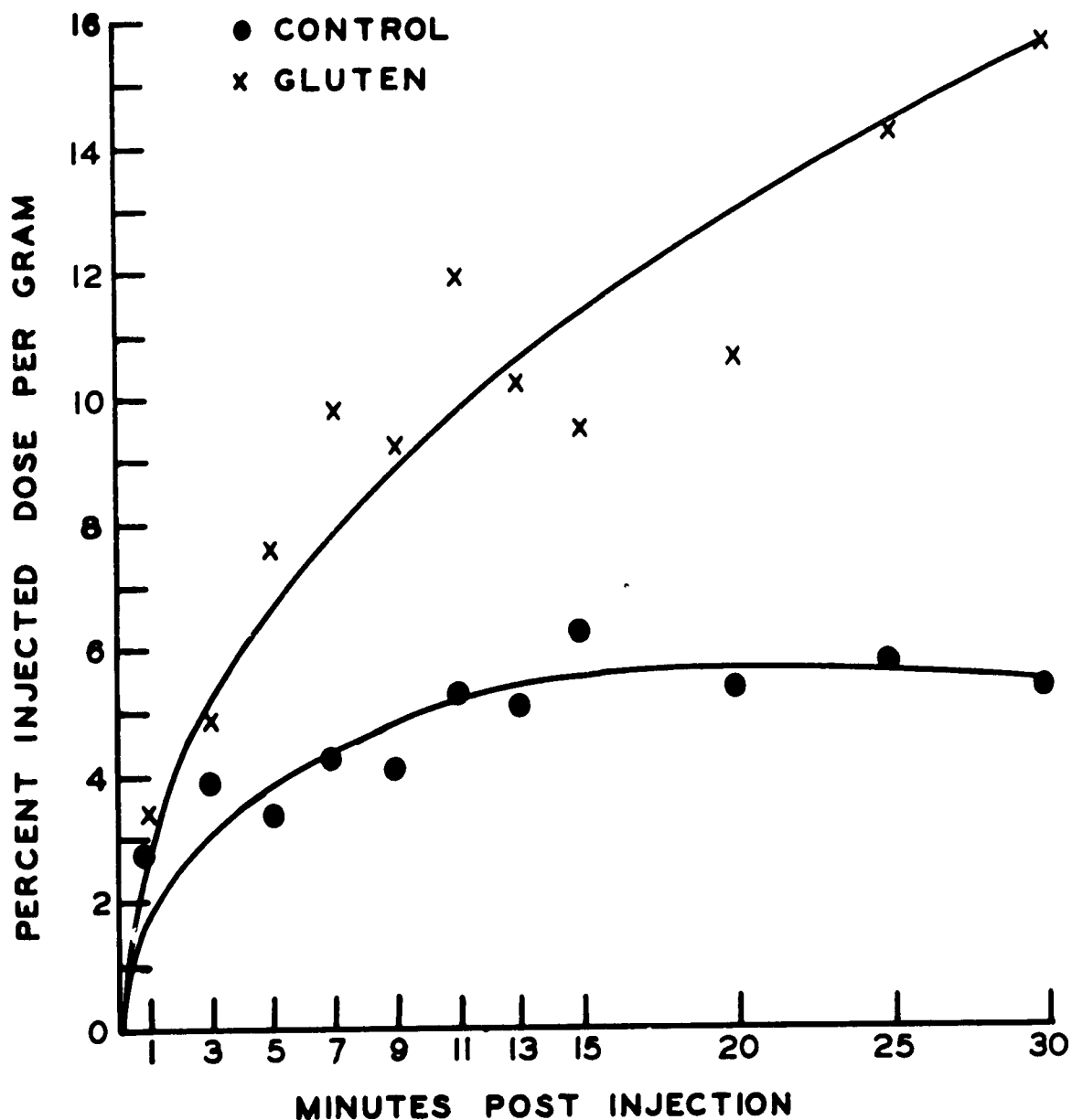


FIGURE 6. RATE OF ACCUMULATION OF COLLOIDAL  $\text{CRP}^{32}\text{O}_4$  IN THE SPLEEN OF CONTROL AND LYSINE DEFICIENT (GLUTEN DIET) RATS.

TABLE VI. EXCRETION OF  $\text{CrP}^{32}\text{O}_4$  FOLLOWING INTRAVENOUS INJECTION OF COLLOID IN CONTROL (C) AND LYSINE-DEFICIENT (G) RATS.

DAY POST- INOCULATION	% OF INJECTED DOSE			
	In urine/ml		In feces/ml	
	Control Diet	Gluten Diet	Control Diet	Gluten Diet
1	.24	.44	.69	.21
2	.003	.03	.66	.39
3	.005	.04	.62	.19
5	.0026	.0011	.27	.18
Total	.063	.128	.56	.24

we postulated, could result primarily from an alteration of the ability of the host to overcome the invading parasite. In this paper we have reported on the activity of the RES and the probable fate of anthrax spores following subcutaneous inoculation into the rat.

It has been demonstrated that there is a decreased rate of loss of colloidal material from the blood of the lysine-deficient animals. Salvidio and Crosby have reported that RES activity could be dependent on the number and activity of the circulating platelets. It was shown above that lysine deficiency was associated with a significantly decreased number of platelets. These two facts could contribute to the decreased colloid clearance by the RES. At the same time, there is a greater concentration of colloid in the liver and spleen of the deficient animals. Individual reports have shown that the colloid is removed by the fixed macrophages of the RES. At first glance, it would seem that the high tissue concentration with a low blood clearance rate was an anomolous situation. However, this could be explained by the inability of the deficient animals to clear from the organ that colloid that had been removed from the blood. If the difference does lie in the inability of an organ to get rid of the colloid, then considering that the liver secretes its products into the gastrointestinal tract, more of the colloid should be found in the feces of the control than of the deficient animal; Table VI illustrates that such is the case. If this mechanism applied to a material that could be metabolized in the RES of the animal, then it might be expected that the metabolic products would be excreted more rapidly in the control than in the deficient

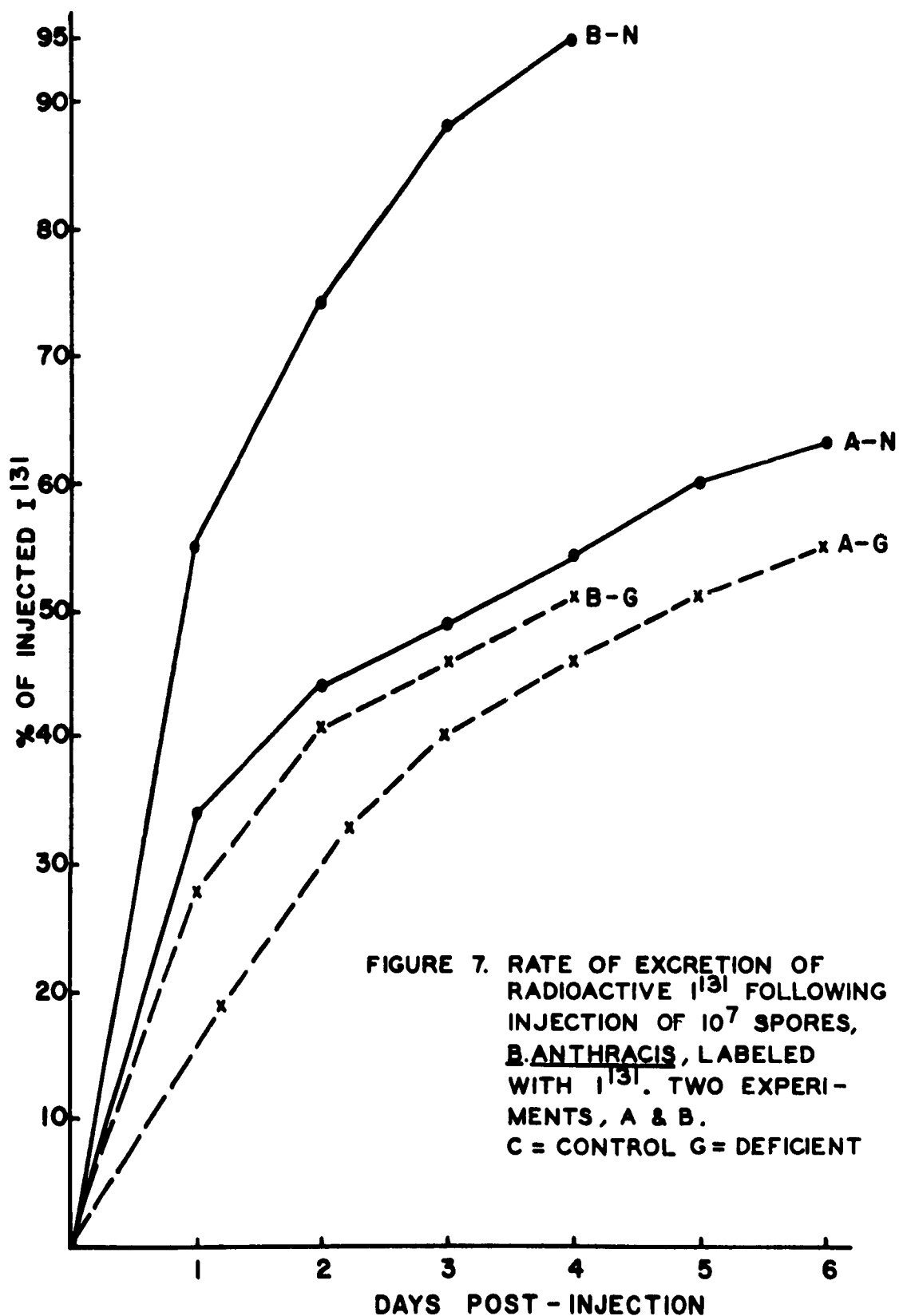


FIGURE 7. RATE OF EXCRETION OF RADIOACTIVE  $I^{131}$  FOLLOWING INJECTION OF  $10^7$  SPORES, B. ANTHRACIS, LABELED WITH  $I^{131}$ . TWO EXPERIMENTS, A & B. C = CONTROL G = DEFICIENT

animals. This is seen in Figure 7. When  $I^{131}$ -labeled spores were injected, dead or living, the greater amount of radioactivity was excreted in the urine by the control animals. Furthermore, for both the colloidal  $CrP^{32}O_4$  in the feces and the  $I^{131}$  in the urine, the significant difference occurred during the first 2 days after the inoculation of the labeled material, the same period during which the maximum mortality rate occurred.

From the foregoing, it is not unreasonable to state that within the time frame of our experiments, the decreased ability of the RES of the host to clear the invading organism from the tissues and subsequently to break down the organism is a major factor of the decreased resistance of the lysine-deficient rats to anthrax.

# ACKNOWLEDGEMENT

The Director and members of the Commission express their sincere appreciation to the participants in the annual meeting. Special appreciation is extended to Mrs. Phebe W. Summers, Mrs. Pearl S. Flohr and Mrs. Marjorie M. Ganley for their assistance in the preparation of this report.